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UNIVERSITY OF ALBERTA

TAXONOMY AND ECOLOGY OF ENDOPHYTIC FUNGI FROM THE MYCORRHIZAE OF ALPINE AND SUBALPINE PLANTS

BY

GODO STOYKE

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MYCOLOGY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

SPRING 1991

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Conidial apparatus of *Phialocephala fortinii*. Asperulate hyphae and small, spherical conidia (1-2 µm diameter). Culture incubated for three months on cereal agar at 4°C. Slide preparation stained with lactofuchsin.

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED "TAXONOMY AND ECOLOGY OF ENDOPHYTIC FUNGI FROM THE MYCORRHIZAE OF ALPINE AND SUBALPINE PLANTS" SUBMITTED BY GODO STOYKE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MYCOLOGY.

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DATE: 24 April 1989

THIS THESIS IS DEDICATED TO
MY PARENTS, HEIDI AND ECKHART STOYKE,
FOR TEACHING ME HOW TO THINK,
AND TO MY SUPERVISOR, RANDY CURRAH,
FOR TEACHING ME HOW TO THINK ABOUT
FUNGI

ABSTRACT

Alpine and subalpine plant communities dominated by dwarf shrub heath were sampled for root endophytic fungi in the Rocky Mountains of Alberta. Sampling yielded 194 fungal isolates obtained from 62 plants belonging to 17 species. Dark endophytes represented 93 % of the isolates and hyaline endophytes 7 %. Only seven % of the 194 isolates sporulated in culture. Two of these fruiting isolates belonged to *Oidiodendron griseum* and were isolated from *Loiseleuria procumbens*, an alpine ericoid shrub. These two isolates represent the only ericoid mycorrhizae observed in the study. The other 11 fruiting strains belonged to *Phialocephala fortinii*, a pathogenic fungus of conifer roots, and were isolated from *Cassiope mertensiana*, *Vaccinium scoparium*, *Arctostaphylos uva-ursi* (Ericaceae) and *Luetkea pectinata* (Rosaceae). Field material revealed the presence of loose wefts of dark, septate hyphae on root surfaces and characteristic conglomerations of irregularly shaped, tightly packed hyphae (sclerotia) in root cortical cells. Morphology of these hyphae was consistent with the morphology of colonies of *P. fortinii* in culture.

Ribosomal DNA of 13 sterile representative isolates of cultural groups was extracted and amplified using the polymerase chain reaction (PCR). Amplified rDNA was restricted using six endonucleases. Correspondence analysis revealed that eight of the 13 sterile isolates clustered close to known isolates of *P. fortinii*, while five sterile isolates formed single-member clusters. Based on the analysis, it was concluded that most isolates were conspecific with *P. fortinii* , which displays a range of cultural morphologies, but is characterized by the presence of mouse-grey (grey to brown) aerial hyphae. All isolates developing sclerotia in culture belonged to *P. fortinii*. Sterile isolates of *P. fortinii* were obtained from *Phyllodoce empetriformis* and *P. glanduliflora* (Ericaceae) as well as from the species listed above.

Axenic inoculation of *Menziesia ferruginea* (Ericaceae) with *P. fortinii* in petri dishes containing cellulose agar led to the formation of loose hyphal wefts and characteristic intracellular cortical sclerotia similar to those observed in roots from field material and similar in appearance to sclerotia formed by apparently mycorrhizal fungi in the European Alps. There was a significant ten-fold increase in seedling mortality of plants inoculated with *P. fortinii* over controls. Plant growth after three months was unaffected by inoculation. *P. fortinii* did not invade the stele of *M. ferruginea*, nor were any other pathogenic effects apparent. *P. fortinii* may be a mycorrhizal partner with plants growing in cold soils with slow decomposition rates. The term "P-type" infection is proposed for this characteristic association of alpine plants.

Key words: alpine mycorrhizae, subalpine, timber-line, P-type infection, *Phialocephala fortinii*, *Menziesia ferruginea*, *Oidiodendron griseum*, RFLP, fungi, Ericaceae.

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1. INTRODUCTION

Mycorrhizae (gr. *mycet-*, =fungus, *rhizus-*, =root) are close associations between the roots of plants and true fungi (*sensu* Kendrick 1985), generally of benefit to both symbionts. At least 87 % of terrestrial plants are mycorrhizal. Benefits of mycorrhizae conferred to the plant by the mycobiont (fungus component of a mycorrhiza) can include increased nitrogen and phosphorus availability, storage of nutrients, drought resistance, protection from heavy metal toxicity, and protection from pathogens. In turn, the fungus is supplied with carbohydrates from the plant, and with habitat. Mycorrhizae not only confer a competitive advantage to plants, but some taxa are obligate mycorrhizal mutualists, unable to grow properly without their fungal partners (Hudson 1986, Lewis 1987, Lalonde and Piché 1988). Mycorrhizae play an important role in the nutrient cycling of terrestrial ecosystems, and some believe that they were of critical importance for the colonization of land by vascular plants during the Ordovician (Pirozynski and Malloch 1975).

Mycorrhizae are classified mostly on the basis of the morphology of the root-fungus association. Ectomycorrhizae form a dense hyphal mantle on the root and establish an intercellular network of hyphae referred to as the Hartig net (Fig. 1-1). Ectomycorrhizal fungi are typically basidiomycetes and are the characteristic mycobionts of temperate woody species. Endomycorrhizal fungi penetrate into cells where they form intercellular and intracellular networks. Endomycorrhizae include a variety of mycorrhizal types, some of which represent forms intermediate to ectomycorrhizae (ectendomycorrhizae). Ectendo-, arbutoid, monotropoid and nyctaginaceous mycorrhizae may display fungal sheaths and intracellular penetration. Vesicular-arbuscular mycorrhizae

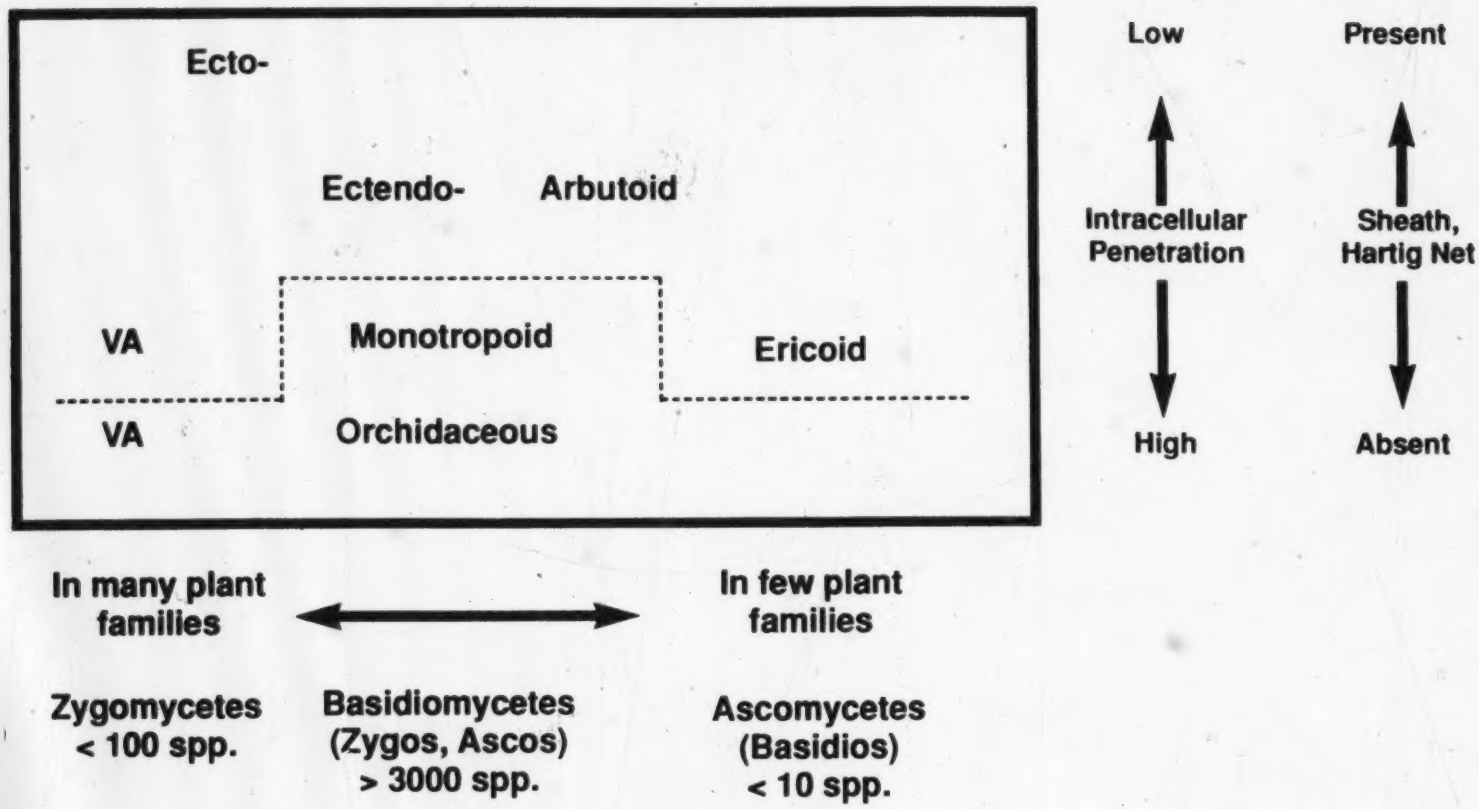

Fig. 1-1. Morphology and ecology of major mycorrhizal types. Mycorrhizal types above dotted line are mutualistic, below dotted line parasitic on the fungus (after Lewis 1987).

(VAM) are the most common mycorrhizal type and are represented in a wide range of plant families. In VAM, hyphae can form finely branched structures (arbuscules) and/or spherical structures (vesicles) inside the host cells. All VAM are formed by fungi classified in the Zygomycota. Orchid mycorrhizae are characterized by intracellular hyphal coils which are digested by the plant. Together with monotropoid mycorrhizae, these are the only mycorrhizae which do not appear to be mutualistic, in both cases the plant parasitizes the fungus. Ericoid mycorrhizae are restricted to a few families of the Ericales (Pyrolaceae, Monotropaceae and Ericaceae, *sensu* Cronquist 1988) and are characterized by dense, hyphal coils in root cortical cells which are not digested by the host. Finally, as yet unclassified mycorrhizae have been described from alpine sedges.

VAM and ectomycorrhizae represent the best-studied types of mycorrhizae because they are present in plants important to agriculture and forestry, respectively. By comparison, other types of mycorrhizae, though of prime importance in natural ecosystems, are less well understood. In particular, the fungi and mycorrhizae of alpine, subalpine and arctic regions are poorly known (Trappe 1988).

The aim of this study was to identify the mycorrhizal fungi of open dwarf-shrub heath meadows at tree line in the Rocky Mountains of Alberta.

The following sections review information on the dominant plant forms at the study sites, members of the Ericaceae, and information on mycorrhizae found in alpine and subalpine zones.

Ericoid Mycorrhizae

Ericoid mycorrhizae are here defined as mycorrhizae in which endophytic fungi form dense intracellular coils within cortical cells of the host root, of benefit to and not digested by the host. A sheath and Hartig net are absent. The fungi are typically dematiaceous, slow-growing and remain sterile in culture (Pearson and Read 1973a).

To date, ericoid mycorrhizae have been described exclusively from vascular plant species belonging to the Ericaceae. Dwarf shrub heath is the dominant form of vegetation in vast areas of subalpine and northern regions of the northern hemisphere (Read 1983). The Ericaceae include common and well known species such as Labrador Tea (*Ledum groenlandicum* Oeder), Bear-berry (*Arctostaphylos uva-ursi* (L.) Spreng.), Lingenberry (*Vaccinium vitis-idaea* L.) and Blueberry (*V. myrtilloides* Michx.). Most of the Ericaceae are calcifuges and prefer acidic soil which is commonly low in nutrients. Ericoid roots are characterized by the lack of root hairs and the presence of fine absorbing rootlets, or "hair roots" (Beijernick 1940 cited in Read 1983). The hair roots have a narrow cortex consisting of one to three layers of large, non-pigmented cortical cells (Hudson 1986).

A few members of the Ericaceae display an ect-endo mycorrhiza characterized by a Hartig net and the occasional presence of a sheath in addition to intracellular coils (Read 1983). This type of mycorrhiza has been termed arbutoid by Harley (1959). However, the majority of Ericaceae display the endomycorrhizal, ericoid (*sensu stricto*) type of infection. Ericoid mycorrhizal fungi form loose wefts of septate mycelium on the surface of the hair roots and

penetrate individual cells, forming dense intracellular coils. Infected cells are often separated by areas of uninfected cells (Harley 1959, Read 1983).

Only six species of ericoid mycorrhizal fungi have been identified to date. The discomycete *Hymenoscyphus ericae* (Leotiales) was described by Read (1974) and has since been shown to form ericoid mycorrhizae in a wide variety of ericoid plants including *Calluna vulgaris*, *Vaccinium angustifolium*, *V. corymbosum*, *V. macrocarpon*, and *Rhododendron* sp. (Bajwa and Read 1986, Bajwa, Abuarghub and Read 1985, Douglas et al. 1989, Duclos and Fortin 1983). Four species of the hyphomycete genus *Oidiodendron* (*O. cerealis*, *O. maius*, *O. rhodogenum* and *O. griseum*) have also been shown to form ericoid mycorrhizae with a variety of hosts belonging to the Ericaceae (Couture et al. 1983, Dalpé 1986, Douglas et al. 1989). Only *Oidiodendron* spp. isolated from ericaceous plants were successful in producing ericoid mycorrhizae while *O. maius* isolated from Sitka spruce and *O. griseum* from wood pulp did not result in mycorrhiza formation. Finally, the basidiomycete *Clavaria* sp. (Clavariaceae) has been implicated as an ericoid mycorrhizal fungus. Reciprocal transfer of nutrients (^{32}P from fungus to host and ^{14}C from host to fungus) can occur. However, it has not been possible to synthesize the association in pure culture due to the difficulty of cultivating *Clavaria* (Englander and Hull 1980, Seviour et al. 1973). Read (1983) questions the mycorrhizal nature of the relationship and suggests that further studies are required to show that the transfer of nutrients is not taking place in the rhizosphere, or that the relationship is actually a mildly parasitic infection which could also involve transfer of nutrients.

Mycorrhiza formation begins with the spread of a network of hyaline, fine (0.5-0.8 µm diameter) hyphae over the root surface (McNabb 1961). Colonization of

the root surface can be observed four weeks after inoculation in cultures of *Rhododendron ponticum* L. and *Hymenoscyphus ericae*. Colonization of most of the root surface was completed after six weeks. However, 100 % mycorrhizal infection in field grown *Vaccinium corymbosum* was only observed in 15 year old plants while three to seven year old plants had infection rates ranging from 58-70 % (Powell and Bates 1981). Penetration of the root begins immediately with colonization of the root surface and is believed to be enzymatic due to the lack of distortion of host wall cellulose microfibrils (Duddridge and Read 1982). Cortical cells are penetrated by hyphal branches entering through the outer wall and then filled by a hyphal coil. Intercellular hyphae as well as infection from one cortical cell to the next appear to be rare. Mature hyphae increase in diameter to 1.5-2.2 µm and develop thicker walls and septa become visible. Mature intracellular hyphae have fewer and less regularly spaced septa, a less regular shape and a diameter one to six times greater than that of external hyphae. Mature hyphae almost completely fill the host cell (McNabb 1961). Hyphae of ericoid mycorrhizal fungi are reported to contribute upwards of 80 % of tissue mass in heavily mycorrhizal regions of the root of some hosts (Read and Stribley 1975 cited in Litten et al. 1985). Besides hyphae with ascomycetous characters (e.g. presence of Woronin bodies), hyphae with dolipore septa have been observed occasionally in hair roots of *Calluna vulgaris* (Bonfante-Fasolo 1980). Even though ultrastructurally the ascomycetous and basidiomycetous hyphae appear similar, Read (1983) questions the mycorrhizal nature of the basidiomycete endophyte.

The intracellular hyphae are enveloped by the host plasmalemma, but separated by a thin, electron-lucent pectic layer, the interfacial matrix. Host cell cytoplasm surrounding hyphae have high concentrations of mitochondria and

ribosomes, suggesting high physiological activity (Read 1983). This condition of presumed high physiological activity appears to predominate during late summer and fall - prime periods of new root formation - in *Vaccinium myrtillus* (Bonfante-Fasolo and Gianinazzi-Pearson 1982).

It was previously believed that mature hyphae are lysed by the host as observed in orchid mycorrhizae, presumably to allow uptake of nutrients by the plant (e.g. Cooke 1977, Harley 1959). However, based on sequential transmission electron micrograqphs (TEMs) it now appears that the host cell cytoplasm degenerates before disintegration of the fungal component. Breakdown of host cell organelles (particularly mitochondria) commences three weeks after infection and is widespread after four weeks (Duddridge and Read 1982). At this stage the hyphae are still completely intact. Degradation of fungal material sets in approximately one week later and is indicated by increased vacuolation (Duddridge and Read 1982). Degradation of plant and fungal intracellular structures leaves behind an empty cortical cell (Read 1983).

Root endophytic fungi isolated from ericaceous plants are generally slow-growing and compete poorly in culture with common fungal soil saprophytes, such as *Trichoderma* sp., *Mucor* sp., *Penicillium expansum* Link ex Gray, *Aspergillus* sp., isolated from the root surfaces of Ericaceae (Pearson and Read 1973a). Hudson (1986) argues that this supports the view of ericoid mycorrhizal fungi as being ecologically obligate biotrophs. However, Pearson and Read (1973a) synthesized typical ericoid mycorrhizae using slow-growing, dark, sterile fungi isolated from soil in which no ericaceous plants are found, indicating that the relationship may not be obligatory for the fungus. Ericoid endophytes tested by Mitchell and Read (1985) grew best on fungus alcohol (meso-inositol) and

simple carbohydrates (glucose, starch), but showed also limited ability to grow on Whatman's cellulose and carboxymethyl cellulose. The opportunistic nature of the mycobiont is further supported by the lack of host specificity generally observed (Pearson and Read 1973a).

A number of studies report growth increase in mycorrhizal plants as compared to non-mycorrhizal plants (e.g. Powell and Bates 1981, Read and Stribley 1973, Stribley and Read 1974). For example, Powell and Bates (1981) inoculated *Vaccinium corymbosum* (Highbush Blueberry) with peat dug from under 80 % infected *V. corymbosum* growing in an unfertilized peat bog. Four year old plants grown with inoculum in the field showed 11-92 % increases in fruit yield as compared to non-inoculated plants, variations in yield increase being dependent on the cultivar used. Reich et al. (1982) did not find a significant correlation between infection intensity and stem growth of two month old *Vaccinium corymbosum*. However, the growth rates were not compared to that of non-mycorrhizal plants (i.e. no control was used) and the identity of the fungi used is unknown.

Ericoid mycorrhizal fungi can use nitrogen sources not available to the plant such as amino acid-N (Bajwa and Read 1986) and proteins (Bajwa et al. 1985). It is interesting to note that the fungus can also use proteins as a source of carbon, presumably reducing the demands the fungus makes on the plant (Bajwa et al. 1985). Transfer of phosphorus from fungus to host and of carbon from host to fungus has been observed by Pearson and Read (1973b).

Alpine and Subalpine Mycorrhizae

Haselwandter and Read (Haselwandter 1979, 1983, 1985, 1986, 1987, Haselwandter and Read 1980, 1982, Read and Haselwandter 1981) have studied alpine and subalpine fungus-root associations at a range of elevations.

Haselwandter and Read (1980) report typical ericoid infection from alpine dwarf shrub heath dominated by ericaceous plants (1900-2200 m). All dominant heath species, e.g. *Vaccinium myrtillus*, display dense hyphal coils within cortical cells referred to as ericoid mycorrhizae. Infection density is higher in *V. myrtillus* plants forming the understory in spruce forests at low elevations (940 m) than in those growing above timberline (1900-2175 m).

Plants of the alpine grass heath (1900-2500 m) were most frequently infected by VAM fungi. However, *Kobresia myosuroides* (Cyperaceae) formed ecto-mycorrhizae with *Cenococcum* while *Carex* displayed associations with the "*Rhizoctonia*" type (Haselwandter and Read 1980). This type is characterized by the production of micro-sclerotia and the occasional presence of hyphal coils in cells. Nival zone vegetation (3100-3200 m) was most frequently infected by fungi of the *Rhizoctonia* type, particularly in plants growing in isolation. Plants growing in high density vegetation patches displayed VA mycorrhizae, but the *Rhizoctonia* type was also frequently present and predominated in some mat forming plants such as *Carex curvula* All.(Haselwandter and Read 1980).

Fungal isolates of the *Rhizoctonia* type were found to benefit *Carex* sp. in laboratory experiments (Haselwandter and Read 1982). Fungi were isolated from surface-sterilized roots of *Carex curvula*, *C. firma* Host and *C.*

sempervirens Vill. No sporulating structures were formed, but the fungi were reported to resemble *Rhizoctonia* and *Phialophora* species. *Carex firma* and *C. sempervirens* seeds were surface-sterilized and grown aseptically in sand with diluted Rorison's mineral nutrient solution in the presence of one of the fungal isolates. Presence of the fungal isolate lead to four-fold increases in shoot and eight-fold increases in root dry weight in *C. firma*. Shoot phosphorous contents were increased two-fold in *C. sempervirens* and more than five-fold in *C. firma*.

Bissett and Parkinson (1979a, b, c) examined the fungal flora of soils at three sites on Mt. Allan in the Alberta Rocky Mountains. The sites examined were a brome (*Bromus*) association at 1900 m, a mountain aven (*Dryas*) association at 2530 m, and a loco-weed (*Oxytropis*) association at 2840 m. Bissett and Parkinson (1979a) employed a soil washing technique for isolating fungi which removes many fungal spores and therefore predisposes the sampling results less towards fungal species producing numerous spores. One-hundred twenty-one fungal species considered common soil saprophytes, including *Mortierella*, *Chrysosporium*, *Phoma*, *Acremonium*, *Cylindrocarpon*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Verticillium* species, were found. In addition, a relatively large number of hyaline and dark sterile species were isolated. The dark (hyaline in brackets) species were isolated from 13 (5), 26 (12), and 14 (11) % of soil particles at the *Bromus*, *Dryas*, and *Oxytropis* sites, respectively. In contrast to the vast majority of other fungal species, the sterile isolates did not show a significant decrease in numbers of isolates with increased sampling depth (samples were taken at 0-2 cm, 5-8 cm, and 15-18 cm, respectively). The total isolation frequency for the fungal population decreased significantly with increasing sampling depth, with isolations being roughly a third less frequent at 15-18 cm than at 0-2 cm on organic particles. On the other hand, sterile hyaline

and dark isolates were encountered roughly twice as frequently at 15-18 cm than at 0-2 cm on organic particles. Bissett and Parkinson (1979b) found that soil depth was the major source of variation in species composition, dark sterile species being the dominant component at the greatest depth (15-18 cm) at all three sites. Ordination of environmental gradients with fungal isolates revealed a predominance of sterile isolates at sites with low temperature, low moisture availability and low potassium levels (Bissett and Parkinson 1979c). Conversely, low soil potassium levels appeared to be the limiting factor for many soil saprophytes only occurring at the soil surface.

Kohn and Stasovski (1990) examined the mycorrhizal status of plants at a high arctic site on Ellesmere Island, Canada. Twenty-four dominant or circumpolar of the 86 plant species observed in the area were examined. Root squashes mounted in glycerin revealed colonization classified as mycorrhizal in 11 of the species. VA-infection was absent except in *Dryopteris fragrans* (L.) Schott (Polypodiaceae). Ecto and ericoid infection was observed in a number of plants while *Cenococcum*-like infection or dark septate hyphae were not observed. However, most plant roots displayed the presence of a hyaline, septate endophyte. The identity of the fungi was not determined.

Currah and Van Dyk (1986) examined the mycorrhizal status of 179 vascular plant species in Alberta. Root segments mounted in glycerin jelly were examined with the light microscope for type of mycorrhizal infection. Dark septate fungi were observed on roots of 6 out of 131 (4.6 %) plant species sampled from boreal forest, aspen parkland, and prairie sites. Thirty-seven of the 47 (78.7 %) plants isolated from alpine, subalpine, or montane sites showed the presence of dark septate fungi. Dark septate fungi isolated from native Alberta orchids

sporulated after lengthy incubation and were identified as *Phialocephala fortinii*, *Leptodontidium orchidicola*, and *Trichocladium opacum* (Currah et al. 1987).

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2. ENDOPHYTIC FUNGI FROM THE MYCORRHIZAE OF ALPINE AND SUBALPINE PLANTS*

Introduction

Ericaceae dominate northern, alpine and subalpine communities in many regions of the northern hemisphere. They form numerous fine, absorbing "hair roots" which are unique in that they lack an epidermis and root hairs and have a narrow cortex of one to three layers of large cortical cells surrounding the vascular cylinder. The cortical cells may contain coils of fungal hyphae which also form loose wefts of hyphae over the surface of the root. When isolated in pure culture, these root-associated fungi form darkly pigmented, slow-growing, sterile colonies. Associations between the hair roots and these fungi are generally referred to as ericoid mycorrhizae (Harley 1959, Read 1983).

Only four species of fungi isolated from ericoid plants and forming ericoid mycorrhizae *in vitro* have been identified. The discomycete *Hymenoscyphus ericae* (Read) Korf and Kernan (Leotiales) (Kernan and Finocchio 1983) was described by Read (1974) as *Pezizella ericae*. It was isolated from *Calluna vulgaris* (L.) Hull. and has been shown to form ericoid mycorrhizae in many ericoid plants including *Calluna vulgaris*, *Vaccinium angustifolium* Ait., *V. corymbosum* L., *V. macrocarpon* Ait., and *Rhododendron* sp. (Bajwa and Read 1986, Bajwa *et al.* 1985, Douglas *et al.* 1989, Duclos and Fortin 1983). Four species of the hyphomycete genus *Oidiodendron* (*O. cerealis* (Thüm.) Barron, *O. maius* Barron, *O. rhodogenum* Robak and *O. griseum* Robak) have also been

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shown to form ericoid mycorrhizae with a variety of ericoid hosts (Couture *et al.* 1983, Dalpé 1986, Douglas *et al.* 1989). Of these, *O. maius* and *O. griseum*, were isolated from ericoid hosts. The hyphomycete *Scytalidium vaccinii* Dalpé, Litten and Sigler isolated from *Vaccinium angustifolium* has been shown to form mycorrhizae *in vitro* (Dalpé *et al.* 1989).

The basidiomycete *Clavaria argillacea* Fr. (Clavariaceae) has also been implicated as an ericoid mycorrhizal fungus (Englander and Hull 1977, 1980, Seviour *et al.* 1973) but no mycorrhizae have been synthesized *in vitro* because of the difficulty in culturing the fungus.

Recently, *Gymnåscella dankalienses* (Castellani) Currah (Gymnoascaceae; *sensu* Currah 1985), the *Oidiodendron* anamorph of *Myxotrichum setosum* (Eidam) Orr, Kuehn and Plunkett and the *Geomyces* anamorph of *Pseudogymnoascus roseus* Raillo (both Myxotrichaceae) have been shown to form ericoid mycorrhizae *in vitro* with *Vaccinium angustifolium* (Dalpé 1989). However, these species have not been isolated from ericoid plants.

Few studies have examined ericoid mycorrhizal fungi in natural ecosystems (e.g. Christoph 1921, Doak 1928, Freisleben 1933, Khan 1972, Singh 1974), fewer still alpine and subalpine ecosystems dominated by Ericaceae (e.g. Haselwandter 1987) and the identity of the fungi involved remains unknown. Our aim was to isolate and identify the fungi forming mycorrhizae within alpine and subalpine plant communities dominated by ericaceous plants at several locations in the Rocky Mountains of Alberta.

Materials and Methods

Samples were obtained from three sites in the Canadian Rocky Mountains. Site 1, in the vicinity of Outpost Lake, Jasper National Park, was sampled on July 26, 1988. It is an open meadow dominated by *Abies lasiocarpa* (Hook.) Nutt., *Cassiope mertensiana* (Bong.) D. Don, *Phyllodoce glanduliflora* (Hook.) Coville, and *P. empetriformis* (Smith) D. Don, at 2010 m above sea level (a.s.l.). Site 2, in the vicinity of Arrowhead Lake, Jasper National Park, was sampled on August 28, 1989. It is a dry ridge at 2000 m a.s.l. dominated by *Abies lasiocarpa* with a dense ground cover of *Loiseleuria procumbens* (L.) Desv. Site 3, a mixed herb meadow at a variety of elevations from 1480 to 2350 m a.s.l. on Mount Allan, Kananaskis Country, was sampled on July 1,1989. Seventeen species were sampled (Table 2-1). Voucher specimens were deposited at the University of Alberta Vascular Plant Herbarium (ALTA), accession numbers ALTA 94078 - 94139.

Plants were excavated with their root systems intact and packed in moist paper towels. Within 24 hours all plants were transported to the laboratory where the roots were separated and washed. From each plant two root samples were prepared: one sample was placed in formalin-acetic acid-alcohol (FAA; 10:35:10:5 formalin - water - ethanol - acetic acid; see below), the other was processed as follows. Root segments five to ten mm in length were submersed in a 20 % solution of household bleach for one minute, and rinsed twice with sterile, distilled water. These were placed onto modified Melin-Norkrans agar (MMN; Marx 1969). After one week, fungi growing from the root samples were transferred to corn-meal agar (CMA; Difco, Detroit, USA). After two months all

Table 2-1. Sterile fungal root endophytes isolated from plants of the Alberta Rocky Mountains.

Plant species (# sampled)	Site	Type of root endophyte[a]
GRAMINEAE		
Unidentified (1)	1	DSE, HSE
CARYOPHYLLACEAE		
Minuartia biflora (2)	3	DSE
SAXIFRAGACEAE		
Saxifraga oppositifolia (1)	3	-
ROSACEAE		
Dryas drummondii (1)	3	-
Luetkea pectinata (5)	1	DSE, HSE
Rubus pedatus (2)	1	DSE
ERICACEAE		
Arctostaphylos uva-ursi (3)	3	DSE
Cassiope mertensiana (12)	1	DSE, HSE
Cassiope tetragona (4)	3	DSE
Loiseleuria procumbens (2)	2	DSE
Phyllodoce empetriformis (2)	1	DSE, HSE
Phyllodoce glanduliflora (10)	1,3	DSE
Vaccinium scoparium (7)	1	DSE
Vaccinium membranaceum (3)	1	DSE
PRIMULACEAE		
Androsace chamaejasme (1)	3	DSE

[a] DSE= dark, septate endophyte, HSE=hyaline, septate endophyte.

Table 2-1. (continued)

Plant species (# sampled)	Site	Type of root endophyte[a]
BORAGINACEAE		
Myosotis alpestris (3)	3	-
ASTERACEAE		
Antennaria lanata (3)	1,3	DSE

strains were transferred to cereal agar (CER; Padhye *et al.* 1973) and oat-meal agar (OAT; Padhye *et al.* 1973) and incubated in the dark at 4°C. Identified sporulating isolates were deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH).

Root samples stored in FAA were washed with distilled water, cleared, stained (Phillips and Hayman 1970) - substituting chlorazol black for trypan blue (Brundrett *et al.* 1984) - and mounted in glycerin jelly to observe the pattern of hyphal growth within the root. Colonies and vegetative mycelia were described from cultures grown on CMA for three months at 21°C. Descriptions of microscopic structures are from samples stained with lactofuchsin and mounted in glycerin jelly.

Menziesia ferruginea J.E. Smith (Ericaceae) and *Phialocephala fortinii* Wang and Wilcox isolated from *Luetkea pectinata* (Pursh) Kuntze (Rosaceae) were used for axenic mycorrhizal synthesis. Seeds of *M. ferruginea* were surface-sterilized (Smreciu and Currah 1989) and placed on tap-water agar (TWA; 20 g agar, 1 l tap water) until germination. The seedlings were then transferred to CER and inoculated with a plug from a fresh culture of *P. fortinii* seven days after transfer. Seven days after inoculation the whole root was excised, mounted in glycerin jelly, and examined with the compound microscope.

Nomenclature of vascular plant species follows Packer (1983).

Results and Discussion

A total of 181 strains of darkly pigmented, simple septate fungi were obtained

(Table 2-1). Sporulating strains were identified as *Phialocephala fortinii* (11 strains; Fig. 2-1) and *Oidiodendron griseum* (two strains; Fig. 2-5). *P. fortinii* sporulated slowly and only after three to six months of incubation at 4°C. *O. griseum* sporulated within seven days at room temperature. Data concerning provenience and disposition of identified strains are given in Table 2-2.

Because of cultural and morphological similarities between many of the non-sporulating, darkly pigmented sterile isolates, and sporulating isolates determined to be *P. fortinii*, it is possible that many of the dark, sterile isolates are *P. fortinii*. Cultural and genetic studies are underway to determine if the sterile strains are related to the named isolates.

Sporulating strains of *P. fortinii* were isolated from *Cassiope mertensiana* and *Arctostaphylos uva-ursi* (L.) Spreng. (Ericaceae), and from *Luetkea pectinata* (Rosaceae) common in subalpine communities at the timber line. When root samples from these plants were cleared, stained and examined with the light microscope, characteristic configurations of darkly pigmented hyphae were observed on the root surfaces and inside cortical cells. Hyphae were 2-4 µm broad, thick-walled, simple septate, occasionally repeatedly branched and contorted, with cells long and narrow or short, swollen and lobed (Figs. 2-2, 2-3, 2-6 and 2-8), and occasionally forming structures resembling sclerotia. These consist of irregularly lobed, closely packed hyphae. A distinctive arrangement of these hyphae was observed in *Luetkea pectinata* (Fig. 2-3). In this plant, darkly pigmented fungal hyphae appeared to fill many of the cortical cells and the pigmented epidermal cells of the root. In some cases, roots of the same plant showed various stages in the development of the sclerotia, from small groups of lobed cells (e.g. Fig. 2-4), larger groups (e.g. Fig. 2-8) to large, roughly globose

Table 2-2. Sporulating fungal root endophytes isolated from subalpine plants of the Alberta Rocky Mountains.

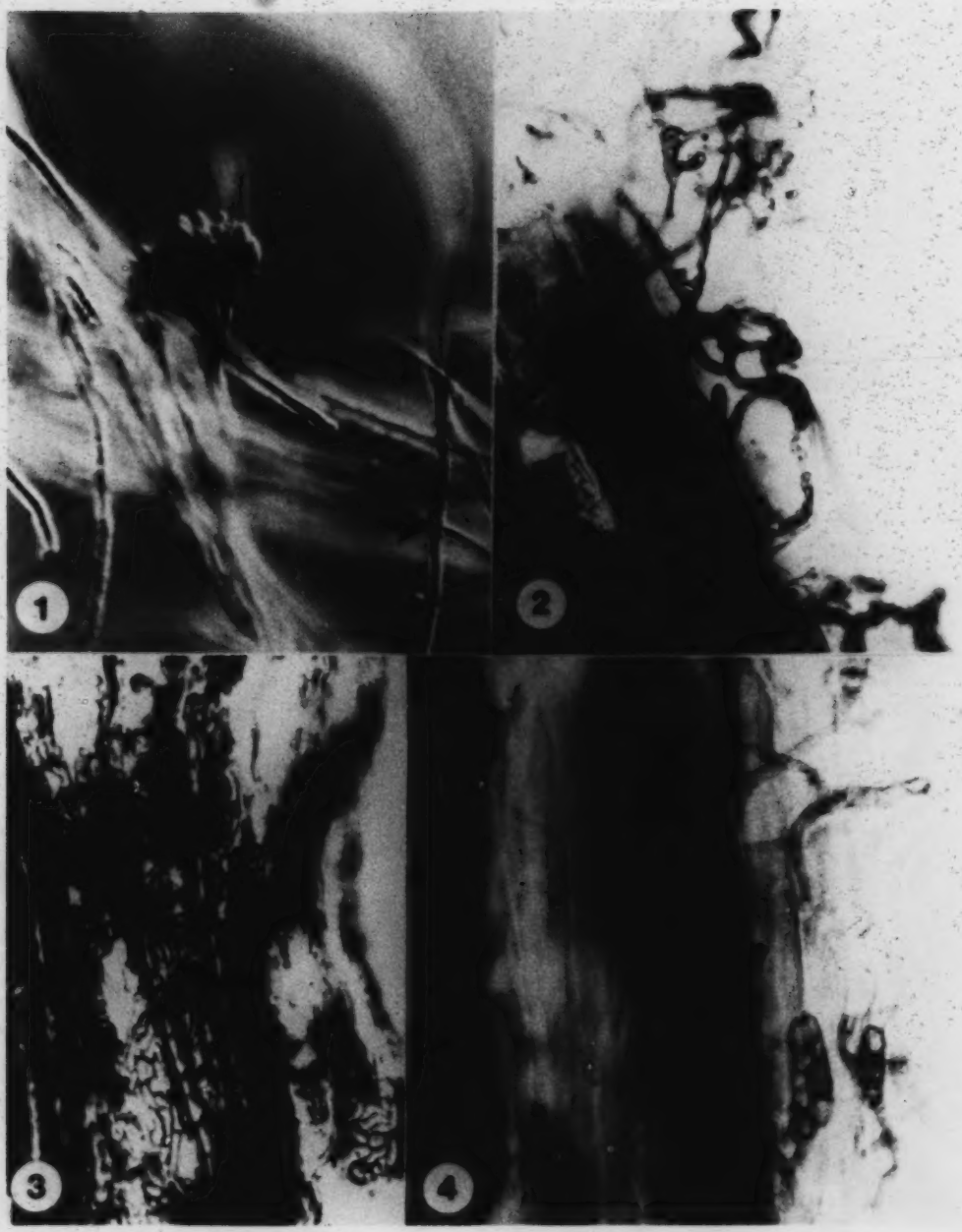
Plant Species	Access. No.	Fungal Species	Access. No.
		Site 1	
Cassiope mertensiana	ALTA 94085	*Phialocephala fortinii*	-
C. mertensiana	ALTA 94091	*P. fortinii*	-
C. mertensiana	ALTA 94094	*P. fortinii*	-
Luetkea pectinata	ALTA 94095	*P. fortinii*	UAMH 6677
L. pectinata	ALTA 94096	*P. fortinii*	-
Vaccinium scoparium	ALTA 94090	*P. fortinii*	-
		Site 2	
Loiseleuria procumbens	ALTA C6514	*Oidiodendron griseum*	UAMH 6514
L. procumbens	-	*O. griseum*	-
		Site 3	
Arctostaphylos uva-ursi	ALTA 94134	*P. fortinii*	-

Figure 2-1. *Phialocephala fortinii.* (UAMH 6677) *ex Luetkea pectinata*, Outpost Lake, Jasper National Park). Large, fan-shaped conidial apparatus and dark, thick-walled, asperulate vegetative hyphae (arrow). From a culture grown on CMA for six months at 4°C. X 560.

Figure 2-2. Dark, simple septate, thick-walled, tortuous hyphae associated with hair root of *Cassiope mertensiana* (source of a confirmed isolate of *P. fortinii*). X 560.

Figure 2-3. Dense intracellular development of dark, thick-walled, irregularly lobed hyphal cells forming sclerotic masses within the cortical cells of *Luetkea pectinata* from Outpost Lake, Jasper National Park (source of *P. fortinii* UAMH 6677). X 560.

Figure 2-4. Root squash prepared from a seven day old axenic culture of a seedling of *Menziesia ferruginea* inoculated with *P. fortinii* (UAMH 6677). Hair root showing some superficial hyphae and a small sclerotial mass of dark, thick-walled, lobed cells. X 560.


1
2
3
4

2-5. *Oidiodendron griseum*. Elongate, rigid, darkly pigmented conidiophores bearing conidia (UAMH 6514 *ex* root of *Loiseleuria procumbens*, Arrowhead Lake, Jasper National Park) From a culture grown on CER for seven days at 21°C. X 1000.

2-6 to 2-9. Root squash preparations of *Loiseleuria procumbens*, Arrowhead Lake (source of *O. griseum* UAMH 6514).

2-6. Hair root showing heavy superficial reticulum of dark, thick-walled tortuous simple septate hyphae. X 420.

2-7. Three cortical cells showing fine, lightly pigmented, intracellular hyphal coils (morphology consistent with *O. griseum*). X 800.

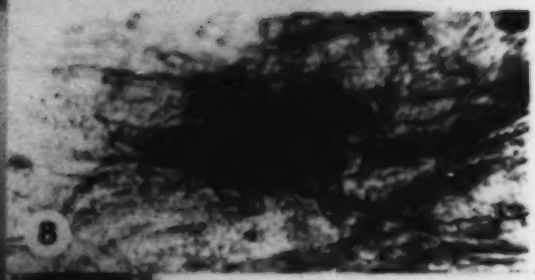
2-8. Dense formation of dark, thick-walled, irregularly lobed cells. X 560.

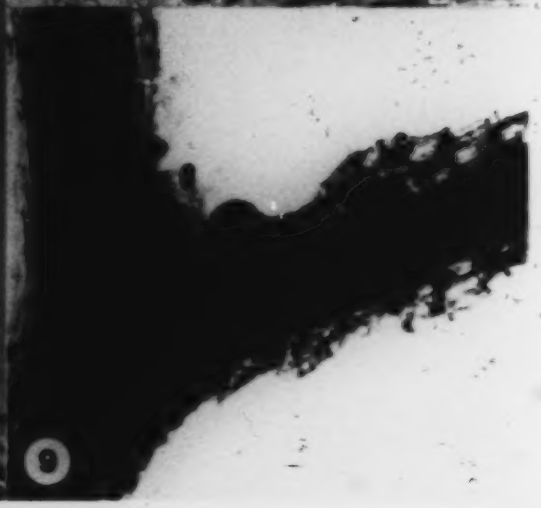
2-9. Large, roughly globose sclerotia of dark, thick-walled, irregularly lobed hyphal cells within hair root. X 120.

5

6

7


8


9

and 30 - 50 µm in diameter, compact masses of darkly pigmented, thick-walled hyphae within the root cortex (Fig. 2-9).

In my opinion, similar sclerotia-forming fungi on and in sampled roots are *P. fortinii*. Axenic reinoculation with confirmed isolates of *P. fortinii* and *Menziesia ferruginea* seedlings yielded mycorrhizae of the type noted in field material (Fig. 2-4) and described above.

Details of colonial and mycelial morphology also support this conclusion. Isolates of *P. fortinii* grown on CMA have submerged, fawn to dark brown hyphae. The simple-septate, faintly to darkly pigmented, smooth to asperulate hyphae grow slowly (0.16 µm/hr) but evenly through the medium except where aggregates of hyphae and monilioid cells form darkly coloured sclerotial conglomerations. The number of sclerotia varies according to strain. In culture, sclerotia can be up to 300 µm in diameter and may be so numerous as to make the reverse of the colony appear speckled.

Field material of *Loiseleuria procumbens* had two distinctive fungi associated within the roots. A type assignable to *P. fortinii* was observed in all cases as was a thinner-walled, less strongly pigmented fungus within the cortical cells (Fig. 2-7). The latter were tentatively identified as *Oidiodendron griseum*. From our samples of *L. procumbens* we isolated *O. griseum* in each case as well as dark, sterile colonies typical of *P. fortinii*. In culture, *O. griseum* differs from *P. fortinii* in having vegetative hyphae which are more lightly pigmented. It also sporulates quickly. I have not yet been able to demonstrate conclusively that the fungus observed in Fig. 2-7 is *O. griseum*. *O. griseum* is widely distributed, primarily in soil enriched with woody debris, litter and decaying plant material (Domsch *et*

al. 1980). It has also been shown to form typical ericoid mycorrhizae with *Vaccinium angustifolium in vitro* (Dalpé 1986).

P. fortinii has been observed as a root endophyte in several studies. Wang and Wilcox (1985) and Wilcox and Wang (1987) found the species to be pathogenic to *Pinus resinosa* Ait. and *Picea rubens* Sarg. seedlings grown with the fungus in monoxenic culture. In *P. resinosa*, a thick fungal mantle with intercellular penetration develops. Dense aggregations of chlamydospores (sclerotia) develop within cells of the cortex and endodermis and considerable lysis occurs in the stele. *P. fortinii* has also been identified in the mycorrhizae of terrestrial orchids (Currah *et al.* 1987, 1988), but its role in these systems is unknown. Currah *et al.* (1988) report sparse hyphae and cortical sclerotia from *Calypso bulbosa* (L.) Oakes which resemble colonization patterns from our field material.

In order to determine if *P. fortinii* could form mycorrhizae *in vitro* and to assess the effects of the fungus in roots we grew seedlings of *Menziesia ferruginea* (Ericaceae) with an isolate of *P. fortinii* and found that at least at the seedling stage, dense colonization of hair roots occurred (Fig. 2-4) without apparent adverse effects on the host. Tests of relative performance of colonized and uncolonized seedlings are now required to determine if *P. fortinii* is a mycorrhizal endophyte.

Some subalpine soils contain relatively coarse organic material and are high in organic acids, lignin and cellulose because of slow decomposition rates (Read 1983). In these environments, some plants develop an apparently mycorrhizal relationship with a dark, septate fungus resembling *Phialocephala fortinii*. This

type of association differs from what is normally referred to as ericoid mycorrhiza by its lack of dense, intracellular hyphal coils and the presence of intracortical sclerotia.

To date, mycorrhizae of the Ericaceae (*sensu stricto*) have been assigned to the arbutoid or the ericoid *sensu stricto* type of association, the latter being predominant (e.g. Dalpé 1986, Dalpé *et al.* 1989, Read 1983). Ericoid mycorrhiza is characterized by a light weft of hyphae on the root surface and extensive and dense coiling of hyphae in the root cortical cells (Read 1983). However, none of the dark isolates examined in our study displayed dense coiling in host root cortical cells of field or lab material. Instead, there develops an extensive weft-like investiture of hyphae which occasionally penetrate the root cortical cells and which often form sclerotia of irregularly lobed hyphal cells. Furthermore, it is clear that the association we are observing in alpine and subalpine habitats is not restricted to ericaceous hosts, but is found in other perennial plants. Dark septate hyphae and sclerotia on the roots of alpine and subalpine plants have also been observed by Currah and Van Dyk (1986), Haselwandter (1987), and Haselwandter and Read (1980) and observed to be more common than vesicular-arbuscular mycorrhizal fungi (VAM) in some zones of the European Alps (Haselwandter 1987, Haselwandter and Read 1980). Haselwandter (1987) found dark, septate hyphal colonization in roots of plants growing at all elevations, but especially in open nival zones. The endophytes were not identified, but were reported to resemble *Rhizoctonia* or *Phialophora* (Haselwandter 1987). We believe that these endophytes may be conspecific or at least congeneric with *P. fortinii*. Axenic inoculation of two *Carex* species with dark endophytes resulted in increased shoot phosphorus levels and shoot and root dry weight (Haselwandter and Read 1982). A study of 179 vascular plant

species in Alberta revealed the presence of dark septate hyphae on the roots of 6 % of low-elevation and 79 % of alpine, subalpine or montane species (Currah and Van Dyk 1986), indicating that this type of colonization is predominantly alpine.

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3. CHARACTERIZATION OF STERILE ENDOPHYTIC FUNGI FROM THE MYCORRHIZAE OF SUBALPINE PLANTS*

Introduction

Strains of dematiaceous, sterile, septate fungi have been isolated repeatedly from the roots of alpine and subalpine plants. These fungi appear to represent a dominant form of high altitude root colonization (Currah and Van Dyk 1986, Haselwandter 1987, Haselwandter and Read 1980, Stoyke and Currah 1991). Of 179 vascular plant species examined , septate fungi were found in association with roots of 6 % of low-altitude and 79 % of alpine, subalpine, and montane species in Alberta (Currah and Van Dyk 1986). A similar pattern of association has also been found to be wide-spread in nival zones of the Austrian Alps. The dark, septate fungi produced micro-sclerotia and occasionally hyphal coils in cells (Haselwandter and Read 1980). Due to absence of spore formation, the fungal endophytes were not identified, but were reported to resemble *Rhizoctonia* or *Phialophora* by Haselwandter (1987). A number of Albertan strains of these dark, sterile fungi have sporulated in culture and have been identified as *Phialocephala fortinii* Wang and Wilcox (Stoyke and Currah 1991).

It is not yet clear whether these root-fungus associations are mycorrhizal. Axenic inoculation of *Carex* with dark isolates by Haselwandter and Read (1982) resulted in increases of shoot phosphorus levels and shoot and root dry weight.

This paper reports on our efforts to identify sterile, septate fungi isolated from

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subalpine mycorrhizae of the Alberta Rocky Mountains using morphology and restriction fragment length polymorphism (RFLP) analysis. Cultural groups were established on the basis of macroscopic characters, such as pigmentation, presence of sclerotia, and distribution of hyphal mats. Restriction fragment analysis was then used to analyze intra- and inter-group genetic variability, and to compare unknown isolates with fungal endophytes of known identity.

Materials and Methods

Samples were collected in the vicinity of Outpost Lake, Jasper National Park, on July 26, 1988, from a subalpine dwarf shrub heath dominated by *Cassiope mertensiana* (Bong.) D. Don, *Phyllodoce glanduliflora* (Hook.) Coville, and *P. empetriformis* (Smith) D. don, and *Abies lasiocarpa* (Hook.) Nutt., at 2010 m a.s.l. Twenty-six plants belonging to five ericoid and four non-ericoid species were sampled (Table 3-1). Nomenclature of vascular plants follows Packer (1983).

Plants were excavated with their root systems intact and packed in moist paper towels. Within 24 hours all plants were transported to the laboratory where the roots were separated and washed. Root segments 5 to 10 mm in length were submersed in a 20 % solution of household bleach for one minute, and rinsed twice with sterile, distilled water. Segments were placed on modified Melin-Norkrans agar (MMN; Marx 1969). After one week, fungi growing from the root samples were transferred to corn-meal agar (CMA; Difco, Detroit, USA).

Fungal endophytes were divided into cultural groups based on colonial morphology on CMA after three months incubation at 21°C. Characters used

Table 3-1. Sterile fungal root endophytes isolated from plants of the Alberta Rocky Mountains and sources of fungi of known identity used for restriction fragment analysis.

Plant species	Isolate (Cultural Group or Species, where known)	Location
Calluna vulgaris	DAOM 185550 (*Hymenoscyphus ericae* Read)	UK
Cassiope mertensiana	2BIP (J), 10Ab, 20a(AG), 13IBb (-), 4b (O), 17IP (T), 10AIP (Y), 11IIAP (P)	CDN
Luetkea pectinata	13IIc (S), 9Ba (Q), 14P, 21aP (AF)	CDN
Phyllodoce empetriformis	18Ic (Q), 18IP(AD)	CDN
Phyllodoce glanduliflora	6B2P (AB), 6Ba (X)	CDN
Pinus sylvestris	FAG-15 (*Phialophora finlandia* Wang and Wilcox)	Finland
Pinus virginiana	28, 155 (*Cenococcum geophilum* Fr.)	USA
Vaccinium scoparium	11Ib (AG), 24c (AD)	CDN
Gramineae	21IIcP (E)	CDN
Decaying wood	DAOM 165556a (*Phialocephala dimorphospora* Kendrick)	USA

were hyphal mat characteristics (texture, zonation, sectoring), presence of sclerotia (masses of short-celled, lobed and closely packed hyphae, 150-300 µm in size), pigmentation, and odour. For clearest observation of distribution of submerged mycelium, cultures were viewed from below. Representative isolates from cultural groups were deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH).

Restriction fragment analysis was performed on 18 representative isolates of the different cultural groups. To aid in classification of the sterile isolates (Table 3-1), restriction fragment patterns were compared to four sporulating isolates representing the following identified species which occur in similar habitats . *Hymenoscyphus ericae* is a known mycorrhizal endophyte of ericoid plants (Read 1974, 1983). *Cenococcum geophilum* can be mycorrhizal with 90 % of alpine trees at tree line and forms ectomycorrhizae with low shrubs (Trappe 1988). *Phialocephala dimorphospora* is congeneric with *P. fortinii*, a species known from habitats at timberline (Stoyke and Currah 1991). *Phialophora finlandia* resembles the sterile endophytes isolated by Haselwandter (1987) from the European Alps. All four species have darkly pigmented hyphae.

Tissue samples for DNA extraction were obtained by placing 12 plugs (3 mm diam.) of mycelium grown on CMA into a liquid medium (15 g malt extract broth, Difco, Detroit, USA, 10 g glucose, 1000 ml tap water) in 250 ml Erlenmeyer flasks. When individual colonies had grown for 2 weeks (diameter approx. 15 mm), they were removed from the broth, filtered, lyophilized and stored in a desiccator at room temperature. Alternatively, 3 mm plugs were taken directly from cultures growing on CMA and then lyophilized. DNA was extracted from tissue samples or plugs using the "mini-prep" method of Zolan and Pukkila (1986).

A portion of the nuclear ribosomal RNA gene repeat (rDNA) was amplified using the polymerase chain reaction (PCR). Two µl of the DNA sample from the mini-prep extraction were added to 98 µl of PCR reaction mixture containing 50 mM each of dATP, dCTP, dGTP, and dTTP, 20 pmol each of primers "A" (5'-TGTACACACCGCCCGTC-3') and "D" (5'-GGAACCTTTCCCCACTTC-3'), 50 mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton x-100, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA) made up to 98 µl with glass-distilled, deionized, autoclaved water. Two drops of sterile mineral oil were layered on top of the mixture to prevent evaporation and condensation during amplification. Samples were amplified in a PTC-100 Programmable Thermal Controller (MJ Research, Chalk River, Ont., CDN) under the following cycling conditions: Cycle 1 (94°C for 3 min., 52°C for 1 min., 72°C for 4 min.); Cycles 2-5 (94°C for 1 min., 52°C for 1 min., 72°C for 4 min.); Cycles 6-29 (94°C for 1 min., 50°C for 1 min., 72°C for 4 min.) and Cycle 30 (94°C for 1 min., 50°C for 1 min., 72°C for 10 min). Samples were checked for absence of secondary PCR products and size by gel electrophoresis, then stored at 4°C (Saike et al. 1988).

Amplified samples were precipitated by mixing with 15 µl of 7.5 M ammonium acetate, followed by addition of 200 µl of cold 95 % ethanol (-20°C). Samples were stored at 4°C for 30 min. to facilitate precipitation and centrifuged at 13 000 rpm for 10 min. at 4°C. The pellet was washed once with 500 µl 70 % ethanol for 10 min., dried in a vacuum desiccator for 15 to 20 min., resuspended in 50 µl of sterile, distilled, deionized water and stored at 4°C.

Amplified DNA was digested using six restriction endonucleases, *Alu*I, *Hae*III,

*Nde*II (Bethesda Research Laboratories, Gaithersburg, MD, USA), *Cfo*I, *Msp*I and *Rsa*I (Boehringer-Mannheim Canada, Laval, Québec, CDN). Seventeen µl of precipitated DNA sample was digested with 5-10 units of enzyme for 3 to 5 hours at 37°C using the buffers supplied by the manufacturers. The digested DNA samples, along with a DNA standard marker (BRL 1 kb ladder, Bethesda Research Laboratories, Gaithersburg, MD, USA), were subjected to electrophoresis on 20 x 20 cm, 0.7 % agarose gels at 1.25 V/cm for 16 h in tris-borate buffer (0.09 M Tris-borate, 0.002 M ethylenediaminetetraacetic acid; EDTA). Gels were stained with ethidium bromide for 10 min., then destained in distilled water for 20 min. before photography.

Primer "A" is homologous with a sequence near the 3' end of the small rRNA subunit; primer "D" is homologous with a sequence near the middle of the large rRNA subunit. The fragment that is amplified by these primers typically ranges from 2-2.2 kb in length and contains the end of the small subunit, the internally transcribed spacers, the 5.8 S subunit, and the beginning of the large subunit. For a review of the structure of the ribosomal DNA in eukaryotes see Gerbi (1986).

Restriction fragment length polymorphisms (RFLPs) were compared among isolates. Presence or absence of bands was coded into a data matrix ("1" for presence, "0" for absence), then analyzed phenetically using the program NTSYS-pc (Rohlf 1987). Ordination was by correspondence analysis. Eigenvectors were extracted from the data matrix using the program CORRESP (NTSYs-pc supplement), then data points were projected onto the first 3 eigenvector axes using the program PROJ (Rohlf 1987).

Results

A total of 127 strains of simple septate fungi were isolated from the different host plants. These isolates were classified into 33 cultural groups (Table 3-2). Cultural groups were established on the basis of macroscopic characteristics (Figs. 3-1 to 3-14). Colonies with hyphae lacking pigmentation (e.g. Figs. 3-1 and 3-2) were assigned to groups A to E. These white colonies were further subdivided on the basis of submerged hyphal mat distribution, e. g. , hyphal mats of colonies belonging to cultural group A had hyphae evenly distributed (Fig. 3-1) while colonies belonging to B displayed uneven hyphal distribution ("sectoring"; Fig. 3-2). Colonies displaying at least some patches of pigmentation (fawn, brown or black) were assigned to groups F to AG. Of the black isolates, all colonies developing sclerotia (black aggregations of submerged hyphae) were placed in groups F to T and colonies lacking sclerotia into U to AG. Colonies displaying sclerotia were distinguished on the basis of sclerotial distribution (occurring throughout the medium, Fig. 3-3, or restricted to narrow, concentric bands, Fig. 3-4), number of sclerotia per square centimeter, and sclerotial size, as well as patterns of distribution of submerged hyphae (even, Fig. 3-7, forming concentric bands, Fig. 3-6, or radially zonate, Fig. 3-3), and of the distribution of aerial hyphae. Dark colonies lacking sclerotia were further subdivided on the basis of hyphal mat branching patterns (Fig. 3-10), zonation (radial or concentric, Fig. 3-14), and presence and distribution of aerial hyphae.

A KEY TO *PHIALOCEPHALA FORTINII* AND CULTURAL GROUPS OF STERILE ENDOPHYTIC FUNGI FROM THE ROCKY MOUNTAINS OF ALBERTA

Characters based on cultures grown on CMA at 21°C for 3 months. "Hyphae", unless noted otherwise, refers to submerged hyphae only:

Table 3-2. Cultural groups of sterile endophytic fungi obtained from the mycorrhizae of alpine plants of the Alberta Rocky Mountains. Hyphal distribution refers to submerged hyphae only unless indicated otherwise. * Group includes isolates of *Phialocephala fortinii*.

Cultural Group	Pigmentation	Distribution of Hyphal Mats	Sclerotial Distribution
A	White	Even	N/A
B	White	Radial branches	N/A
C	White	Uneven, r.b.[1] at periphery absent	N/A
D	White	Uneven, r.b. at periphery present, separated	N/A
E	White	Uneven, r.b. at periphery present, not separ.	N/A
F	Bwn[2]-Bck[3]	R.b. arising from colony centre	Abundant except at centre and periphery
G	Bwn-Bck	R.b. not arising from colony centre, agar stained bluish-gray, radially symmetrical	Restricted to wide concentric band
H	Bwn-Bck	R.b. not arising from colony centre, not radially symmetrical	Restricted to irregular concentric band
I	Fawn	Concentrically zonate	Scarce, restricted to well defined concentric band 3 mm in width
J*	Fawn-Bwn	Concentrically zonate	Scarce and small (~ 150 µm diam.), restricted to concentric band

[1] = radial branches, [2] = brown, [3] = black, [4] = radial branches,

Table 3-2. (continued)

Cultural Group	Pigmentation	Distribution of Hyphal Mats	Sclerotial Distribution
K	Bwn	Even, staining agar bluish-gray	Abundant and large (~ 300 µm diam.), restricted to well defined concentric band
L	Bwn	Even, staining agar bluish-gray	Restricted to well defined concentric band
M	Dark Bwn	Well-defined concentrically multizonate	Restricted to concentric band beginning 15 mm from centre of colony
N	Dark Bwn-Bck	Numerous tangentially oriented hyphae (woolly appearance)	Scarce and small (~ 150 µm diam.), restricted to concentric band beginning 30 mm from centre of colony
O*	Dark Bwn-Bck	Even	Abundant and large (~ 300 µm diam.), restricted to +/- well defined concentric band
P*	Fawn	Radially zonate, aerial hyphae form irregular tufts	Restricted to concentric band beginning 25 mm from centre of colony
Q*	Gr[4]-light Fawn	May be faintly radially zonate, aerial hyphae distributed evenly	Restricted to concentric band beginning 25 mm from centre of colony. ≥30 sclerotia/cm2
R*	Gr- fawn	May be faintly radially zonate, aerial hyphae distributed evenly	Restricted to concentric band beginning 25 mm from centre of colony. ≤20 sclerotia/cm2. Some isolates with large (~ 300 µm diam.) sclerotia.

Table 3-2. (continued)

Cultural Group	Pigmentation	Distribution of Hyphal Mats	Sclerotial Distribution
S*	Bwn-Bck	Even	Restricted to two concentric bands
T*	Bwn-Bck	Even	Unevenly throughout
U	White w/ Bck patches	May be concentrically zonate	N/A
V	Bwn-Bck	Even, aerial hyphae in concentric band	N/A
W	Gr-Bck	Irregular patch of dark central hyphae	N/A
X*	Dark Bwn-Bck	Regular feathery and root-like radial branches	N/A
Y	Dark Bwn-Bck	Irregular thick radial branches	N/A
Z	Gr-Bwn	Concentrically zonate, radial branches	N/A
AA	Gr-Bwn	Few (≤4) radial branches	N/A
AB	Gr-Bwn	Numerous (≥10) radial branches	N/A
AC	Bwn-Bck	Radially and concentrically zonate	N/A

Table 3-2. (continued)

Cultural Group	Pigmentation	Distribution of Hyphal Mats	Sclerotial Distribution
AD*	Reddish to Grayish Bwn	Radially zonate	N/A
AE*	Bwn	Concentrically zonate, aerial hyphae present	N/A
AF	Gr-very faint Bwn	Concentrically zonate, aerial hyphae absent	N/A
AG*	Bwn-Bck	Even, aerial hyphae distributed evenly	N/A

Figs. 3-1 to 3-14. Representative isolates of 13 cultural groups. All cultures grown on corn-meal agar (CMA) at room temperature. The left portion of each figure represents the aerial view, the right portion a view of the same culture from below. X 0.55.

Figs. 3-1. Isolate 21IIaP, group A, 7 wks.

Figs. 3-2. Isolate 2B2P, group B, 7 wks.

Figs. 3-3. Isolate 4P, group F, 7 wks.

Figs. 3-4. Isolate 25Ia, group K, 2 mo.

Figs. 3-5. Isolate 6Bc, group Q, 7 mo.

Figs. 3-6. Isolate 14c, group A, 6 mo.

Figs. 3-7. Isolate 2Ba, group R, 7 mo.

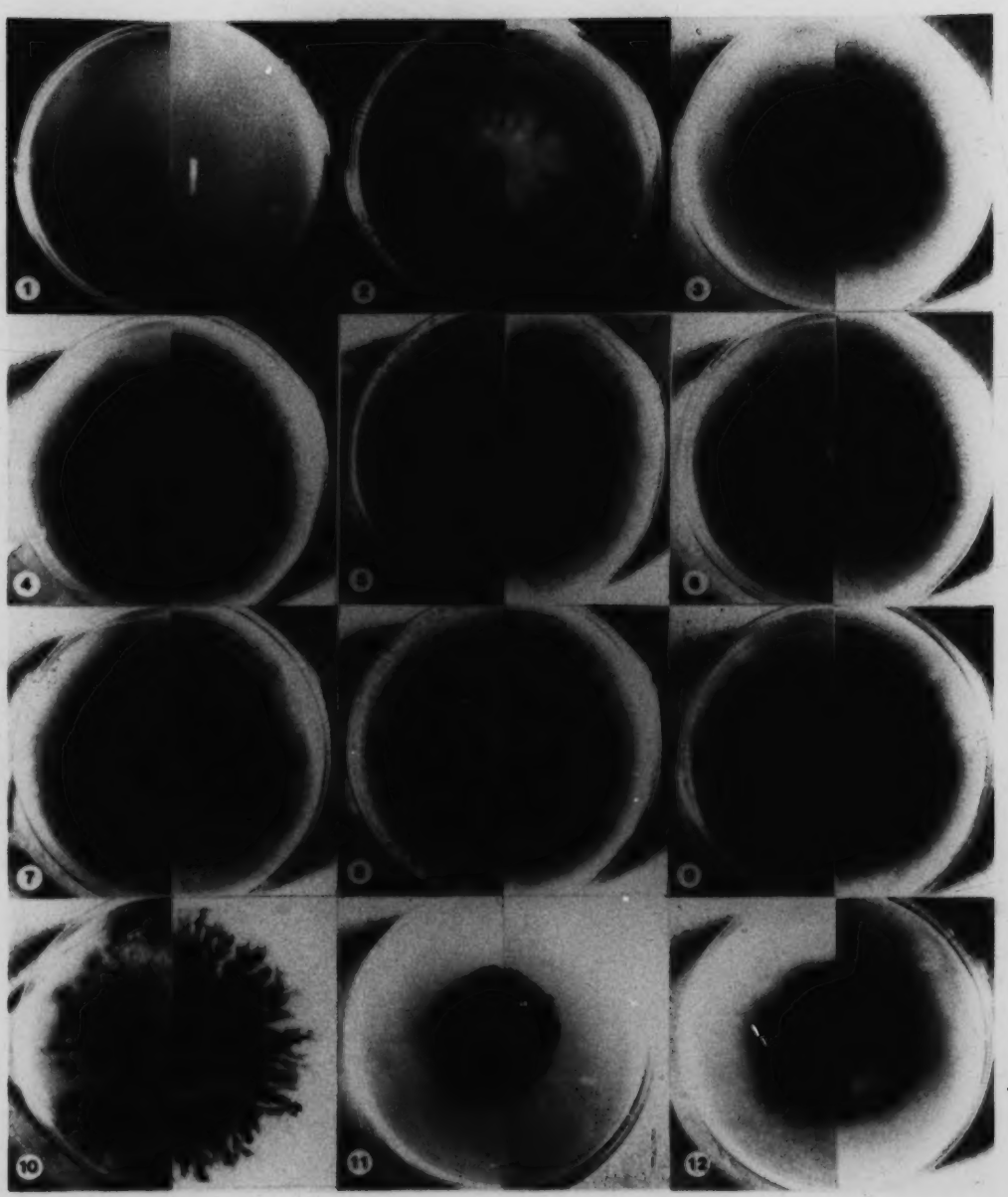
Figs. 3-8. Isolate 20-IP, group V, 7 wks.

Figs. 3-9. Isolate 5Bc, group W, 2 mo.

Figs. 3-10. Isolate 6Ba, group X, 2 mo. Bottom view of a culture grown for 3 mo., X 0.8.

Figs. 3-11. Isolate 10AIP, group Y, 7 wks.

Figs. 3-12. Isolate 13IAP, group A, 7 wks.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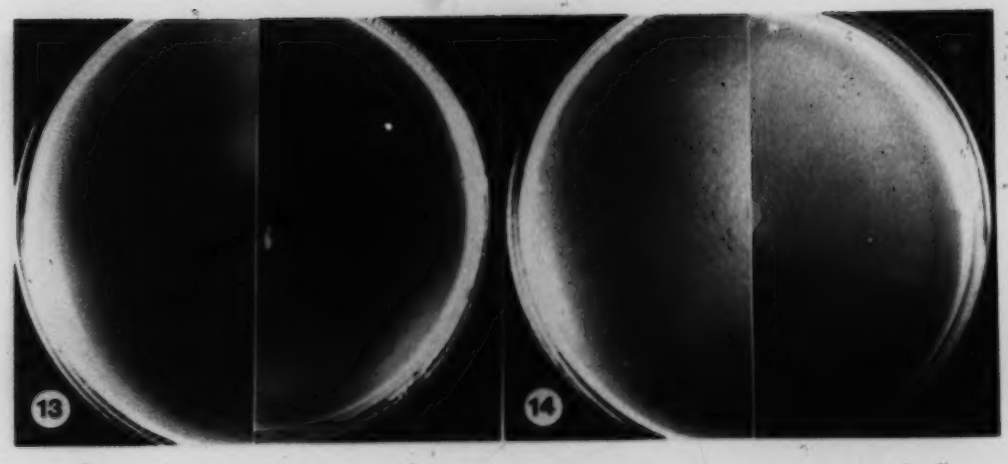
Figs. 3-1 to 3-14. Representative isolates of 13 cultural groups (continued).

Figs. 3-13. Isolate 14P, group AF, 7 wks.

Figs. 3-13. Isolate 21aP, group AF, 7 wks.


13
14

1.a Mycelium white .....Group E

1.b Mycelium with distinct patches of brown, grey or black hyphae.....2

2.a (1.b) Sclerotia present.....

.....Groups J, O, P, Q, R, S, T (*Phialocephala fortinii*)

2.b Sclerotia absent.....3

3.a (2.b) Hyphae forming radial branches.....4

3.b Hyphae not forming radial branches.....6

4.a (3.b) Hyphae grey to brown .....Group AB

4.b Hyphae dark brown to black 5

5.a (4.b) Radial hyphal branches regular, feathery, thin and root-like.....Group X (*Phialocephala fortinii*)

4.b Radial hyphal branches irregular, thick, not feathery and root-like.....Group Y

6.a (3.b) Colony radially zonate or even, if concentrically zonate mycelium brown ..... Groups AD, AE, AG (*Phialocephala fortinii*)

6.b Colony concentrically zonate, mycelium grey to very faint brown.....7

7.a (6.b) Concentric zonation of colony faint, mycelium very fain brown .....

.....Isolate 14P (Group AF)

7.b Concentric zonation of colony distinct, mycelium grey .....

.....Isolate 21aP (Group AF)

Descriptions of Cultural Groups

Cultural Group A

SUBMERGED HYPHAE: white, hyphae evenly distributed throughout the medium.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G1IIP, G9BP2 ex *Luetkea pectinata*, G3IBa, G3IBcP ex *Phyllodoce empetriformis*, G12IIaP ex *Cassiope mertensiana*.

Cultural Group B

SUBMERGED HYPHAE: white, hyphal distribution uneven; hyphae forming radial branches, originating from the centre of the colony.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G2B2P, G2AP ex *Cassiope mertensiana*.

Cultural Group C

SUBMERGED HYPHAE: white, hyphal distribution uneven, radial hyphal branches at periphery of colony absent.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G26P ex *Vaccinium* sp.

Cultural Group D

SUBMERGED HYPHAE: white, hyphae form small, radial branches at periphery of colony, hyphal branches separated by a distinct space.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G5Ba ex *Cassiope mertensiana*.

Cultural Group E

SUBMERGED HYPHAE: white, forming radial hyphal branches at periphery of colony, radial hyphal branches at periphery of colony not separated by a distinct space.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G21IIc ex unidentified Gramineae.

Cultural Group F

SUBMERGED HYPHAE: brown to black, radial hyphal branches arising from centre of colony.

AERIAL HYPHAE: brown, distributed evenly over the surface of the medium

SCLEROTIA: abundant throughout submerged mycelium except at center and periphery of colony.

ODOUR: absent.

STRAINS: G4P ex *Cassiope mertensiana*.

Cultural Group G

SUBMERGED HYPHAE: brown to black, forming radial hyphal branches not arising from centre of colony, agar stained bluish-gray, somewhat opaque. Colonial morphology radially symmetrical .

AERIAL HYPHAE: forming well developed, distinct, grayish concentric band.

SCLEROTIA: sclerotia restricted to wide concentric band.

ODOUR: faintly musty.

STRAINS: G8Bc ex *Phyllodoce glanduliflora*.

Cultural Group H

SUBMERGED HYPHAE: form radial hyphal branches not arising from centre of colony, colonial morphology not radially symmetrical .

AERIAL HYPHAE: grey, distributed evenly over the surface of the medium.

SCLEROTIA: restricted to irregular concentric band.

ODOUR: absent.

STRAINS: G16a ex *Rubus pedatus*.

Cultural Group I

SUBMERGED HYPHAE: fawn, concentrically zonate.

AERIAL HYPHAE: gray, distributed evenly over the surface of the medium.

SCLEROTIA: sclerotia scarce, restricted to narrow (three mm wide) and very well defined concentric band

ODOUR: absent.

STRAINS: G25IIa ex *Cassiope mertensiana*.

Cultural Group J

SUBMERGED HYPHAE: fawn to brown, concentrically zonate.

AERIAL HYPHAE: grey to brown, prominent.

SCLEROTIA: sclerotia scarce and small (approx. 150 µm), restricted to single concentric band.

ODOUR: absent.

STRAINS: G2B1P ex *Cassiope mertensiana*.

Cultural Group K

SUBMERGED HYPHAE: brown, staining agar bluish-gray, opaque.

AERIAL HYPHAE: light brown, prominent

SCLEROTIA: abundant and relatively large (approx. 300 µm).

ODOUR: absent.

STRAINS: G25Ia, G25Ib ex *Vaccinium scoparium*.

Cultural Group L

SUBMERGED HYPHAE: stain agar bluish-gray, opaque.

AERIAL HYPHAE: prominent.

SCLEROTIA: restricted to single concentric band.

ODOUR: absent.

STRAINS: G20b ex *Cassiope mertensiana*.

Cultural Group M

SUBMERGED HYPHAE: dark brown, well-defined concentrically multizonate.

AERIAL HYPHAE: brown, forming loose irregular tufts.

SCLEROTIA: restricted to single concentric band beginning 15 mm from center of colony.

ODOUR: absent.

STRAINS: G15P ex *Vaccinium* sp.

Cultural Group N

SUBMERGED HYPHAE: dark brown to black, large number of tangentially oriented hyphae giving "woolly" appearance to submerged portion of mycelium.

AERIAL HYPHAE: grayish brown, closely appressed.

SCLEROTIA: scarce and small (approx. 150 µm, barely visible with the naked eye), restricted to single concentric band beginning 30 mm from center of colony.

ODOUR: absent.

STRAINS: G6Bc ex *Phyllodoce glanduliflora*.

Cultural Group O

SUBMERGED HYPHAE: dark brown to black, evenly distributed.

AERIAL HYPHAE: grayish brown, in irregular tufts.

SCLEROTIA: numerous and relatively large (approx. 300 µm, easily seen with the naked eye), restricted to single, concentric band

ODOUR: absent.

STRAINS: G4b, G4c ex *Cassiope mertensiana*, G18IBP ex *Phyllodoce empetriformis*.

Cultural Group P

SUBMERGED HYPHAE: fawn, radially zonate.

AERIAL HYPHAE: grayish-brown, forming irregular tufts.

SCLEROTIA: restricted to single concentric band beginning 25 mm from center of colony.

ODOUR: absent.

STRAINS: G11IP ex *Vaccinium scoparium*, G11IIAP ex *Cassiope mertensiana*.

Cultural Group Q

SUBMERGED HYPHAE: grey to light fawn, slightly radially zonate.

AERIAL HYPHAE: brown, distributed evenly over the surface of the medium.

SCLEROTIA: restricted to single concentric band beginning 25 mm from center of colony. Thirty or more sclerotia /cm^2 in sclerotial band.

ODOUR: absent.

STRAINS: G25IIb, G25IIc, G25IIP, G2AbP, G2AcP, G2Bc ex *Cassiope mertensiana*, G18Ic, G19IBbP ex *Phyllodoce empetriformis*, G25IP, G25Ic ex *Vaccinium scoparium*, G9Ba, G14c ex *Luetkea pectinata*, G16c ex *Rubus pedatus*, G19c ex *Phyllodoce glanduliflora*.

Cultural Group R : *Phialocephala fortinii*

SUBMERGED HYPHAE: grey to fawn.

AERIAL HYPHAE: gray to brown, distributed evenly over the surface of the medium. Conidial apparatus forming after 3 to 6 months of incubation at 4°C.

SCLEROTIA: restricted to single, more or less well defined concentric band. Large (more than 300 µm) sclerotia present in some strains. Twenty or fewer sclerotia /cm^2 in sclerotial band.

ODOUR: usually absent.

STRAINS: G2IAa, G2Ba, G2Bb, G11IIBb, G13IAa, G13IAb, G13IAc, G13IBa, G22a, G22-1P, G25IIa ex *Cassiope mertensiana*, G9BP1, G14d ex *Luetkea pectinata*, G8Bb G. G18IB1P ex *Phyllodoce empetriformis*.

Cultural Group S : *Phialocephala fortinii*

SUBMERGED HYPHAE: brown to black.

AERIAL HYPHAE: gray to brown, distributed evenly over the surface of the medium. Conidial apparatus forming after 3 to 6 months of incubation at 4°C.

SCLEROTIA: in two concentric sclerotial bands.

ODOUR: absent.

STRAINS: G13IBb, G13IBc ex *Cassiope mertensiana*, G13IIc ex *Luetkea pectinata*.

Cultural Group T

SUBMERGED HYPHAE: distributed evenly.

AERIAL HYPHAE: gray, distributed evenly over the surface of the medium.

SCLEROTIA: distributed unevenly throughout the submerged portion of the mycelium.

ODOUR: absent.

STRAINS: G11IIBc, G11IIBa, G17IP, G22-2P ex *Cassiope mertensiana*, G15b, G15c ex *Vaccinium* sp, G13IIb, G17IIP ex *Luetkea pectinata*, G11Ic ex *Vaccinium scoparium*, G8Ba ex *Phyllodoce glanduliflora*.

Cultural Group U

SUBMERGED HYPHAE: mostly white, some patches of dark hyphae. May be concentrically zonate.

AERIAL HYPHAE: white, if present.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G1IIb, G3IIc ex *Luetkea pectinata*, G1I ex *Cassiope mertensiana*.

Cultural Group V

SUBMERGED HYPHAE: brown to black.

AERIAL HYPHAE: grey, forming well-developed and distinct concentric band.

SCLEROTIA: absent.

ODOUR: absent or faintly musty.

STRAINS: G8BP ex *Phyllodoce glanduliflora*, G20c, G20BP, G20-1P, G20-2P ex *Cassiope mertensiana*.

Cultural Group W

SUBMERGED HYPHAE: gray to black, central hyphae form irregular dark patch.

AERIAL HYPHAE: gray, distributed evenly over the surface of the medium.

SCLEROTIA: absent.

ODOUR: absent.

STRAINS: G5Ab, G5Aa, G5AP, G5Bc ex *Cassiope mertensiana*, G6Ac, G6Ab, G6Bb ex *Phyllodoce glanduliflora*, G12Ib ex *Antennaria lanata*.

Cultural Group X

SUBMERGED HYPHAE: dark brown to black, forming distinct, regular, feathery, thin and root-like radial branches.

AERIAL HYPHAE: gray.

SCLEROTIA: absent.

ODOUR: faint musty.

STRAINS: G6Ba ex *Phyllodoce glanduliflora*.

Cultural Group Y

SUBMERGED HYPHAE: dark brown to black, forming irregular, thick radial hyphal branches.

AERIAL HYPHAE: faint, forming irregular white patches.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G10AP, G10A1P ex *Cassiope mertensiana*.

Cultural Group Z

SUBMERGED HYPHAE: gray to brown, distinctly concentrically zonate, hyphae forming radial branches.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G10A2P ex *Cassiope mertensiana*.

Cultural Group AA

SUBMERGED HYPHAE: gray to brown, hyphae forming few (four or less) radial branches.

AERIAL HYPHAE: faint, forming irregular white patches.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G13IB1P ex *Cassiope mertensiana*.

Cultural Group AB

SUBMERGED HYPHAE: grey to brown, hyphae forming numerous (ten or more) radial branches.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G6B2P, G6A2P, G6A1P ex *Phyllodoce glanduliflora*, G3IIIb, G20Ba ex *Cassiope mertensiana*, G3IAP ex *Phyllodoce empetriformis*.

Cultural Group AC

SUBMERGED HYPHAE: brown to black, distinctly radially and distinctly concentrically zonate.

AERIAL HYPHAE: grey to brown, distributed evenly over the surface of the medium.

SCLEROTIA: absent.

ODOUR: absent.

STRAINS: G9Ab ex *Luetkea pectinata*.

Cultural Group AD

SUBMERGED HYPHAE: reddish to grayish brown, distinctly radially zonate.

AERIAL HYPHAE: gray, distributed evenly over the surface of the medium.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G18IP ex *Phyllodoce empetriformis*, G18IIP ex *Luetkea*

pectinata, G24c, G24P, G24b ex *Vaccinium* sp.

Cultural Group AE

SUBMERGED HYPHAE: brown, concentrically zonate.

AERIAL HYPHAE: brown or gray to black, distributed evenly over the surface of the medium.

SCLEROTIA: absent.

ODOUR: absent.

STRAINS: G11IIBP, G11IIAc, G23-1P, G1Ia, G3IIIc, G13IAP ex *Cassiope mertensiana*, G8Ab, G8Ac, G8A2P ex *Phyllodoce glanduliflora*, G21IIbP ex unidentified Gramineae.

Cultural Group AF

SUBMERGED HYPHAE: gray to very faint brown, concentrically zonate.

AERIAL HYPHAE: absent.

SCLEROTIA: absent.

ODOUR: musty.

STRAINS: G18IBc2 ex *Phyllodoce empetriformis*, G21P, G21aP, G21Ib, G14b, G14P ex *Luetkea pectinata*.

Cultural Group AG

SUBMERGED HYPHAE: brown to black, lacking any pattern of distinctive hyphal distribution.

AERIAL HYPHAE: gray, distributed evenly over the surface of the medium.

SCLEROTIA: absent.

ODOUR: absent.

STRAINS: G10Ab, G23c, G23d, G2IIIP, G20a ex *Cassiope mertensiana*, G19b, G8A1P, G6Aa ex *Phyllodoce glanduliflora*, G9AP, G9Ac ex *Luetkea pectinata*, G11Ia, G11Ib ex *Vaccinium* sp.

Comparison of sterile isolates with fungal endophytes of known identity

Restriction fragment length polymorphism data were obtained for 18 isolates with 48 distinct bands for 864 data points (Figs. 3-15 to 3-20). Ordination showed a tight cluster of 8 sterile isolates around *Phialocephala fortinii* as well as the presence of several isolates which are very distinct from *P. fortinii* (Fig. 3-21). *Cenococcum geophilum*, which restricted poorly, was distinct for the two enzymes from which results were obtained (Figs. 3-15to 3-20).

The first three axes accounted for 24.8, 15.9, and 14.7 %, respectively, of the variation observed. Eight of the 13 sterile isolates examined through restriction fragment analysis formed a distinct cluster around the two confirmed *Phialocephala fortinii* isolates G13IIc and G13Ib (Fig. 3-21). Overlap of bands was typically around 94 %. The relatively high overall similarity of the cluster suggests that these isolates are conspecific and that the majority of the fungi isolated from the subalpine community sampled belongs to *P. fortinii*. Overlap between members of the *P. fortinii* cluster and other sterile isolates was typically around 67 %. Another known taxon of *Phialocephala* , *P. dimorphospora*, formed a separate and distinct, single member cluster. *P. dimorphospora* is the closest isolate to the *P. fortinii* cluster and differs from *P. fortinii* only on axis 2. Overlap between bands of *P. fortinii* and *P. dimorphospora* was 78 %. None of the isolates resembled *Hymenoscyphus ericae* (Fig. 3-21), which is a known endophyte of ericoid plants (Read 1983). One isolate, 21IIcP, appeared close to *Phialophora finlandia* in the ordination. It is doubtful, however, that 21IIcP is conspecific or congeneric with *P. finlandia* since cultural morphologies of the two

Figs. 3-15 to 3-20. Fluorescent bands of rDNA obtained after PCR amplification and digestion with restriction endonucleases.

Fig. 3-15. *Alu*I. Isolates from left to right: *Phialophora finlandia*, *C. geophilum* 28, *Cenococcum geophilum* 155, *Hymenoscyphus ericae*, *Phialocephala dimorphospora*, 24c, 21IIcP, 21aP, 20a, 18IP, 18Ic, 17IP, 14P, 13IIc, 13Ib, 11IIAP, 11Ib, 10Ab, 10AIP, 9Ba, 6B2P, 4b, 2BIP.

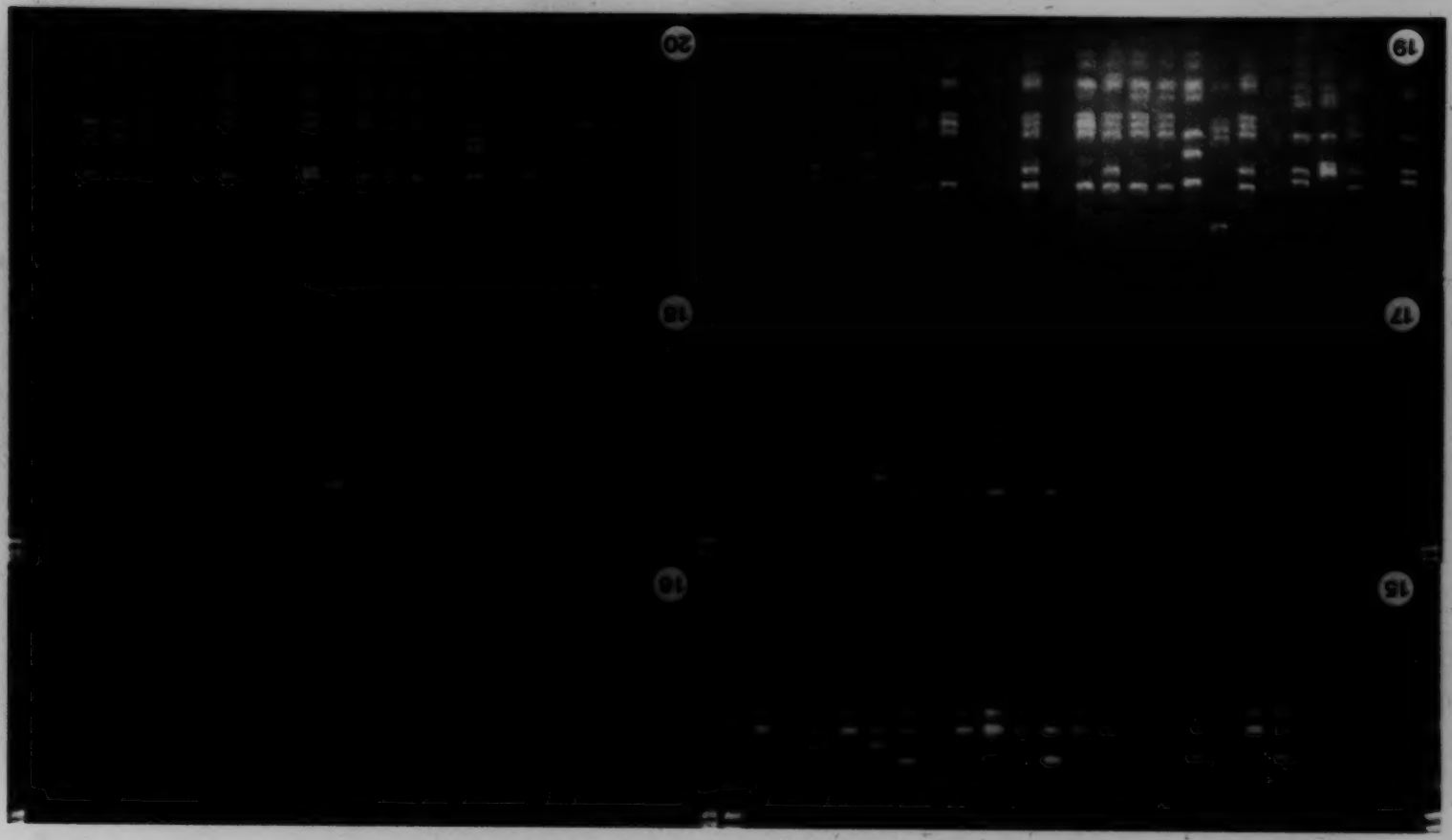
Fig. 3-16. *Msp*I. For sequence of isolates see Fig. 3-15.

Fig. 3-17. *Cfo*I. For sequence of isolates see Fig. 3-15.

Fig. 3-18. *Rsa*I. For sequence of isolates see Fig. 3-15.

Fig. 3-19. *Hae*III. Isolates from left to right: 21IIcP, 6Ba, 11IIAP, 10AIP, 6B2P, 18IP, 24c, 21aP, 14P, 20a, 10Ab, 11Ib, 2BIP, 18Ic, 9Ba, 17IP, 4b, 13Ib, 13IIc, *Phialocephala fortinii*, *Phialocephala dimorphospora*, *Phialophora finlandia*, *Hymenoscyphus ericae*, *Cenococcum geophilum* 28, *C. geophilum* 155. Arranged in order of presumed taxonomic affinity.

Fig. 3-20. *Nde*II. For sequence of isolates see Fig. 3-19.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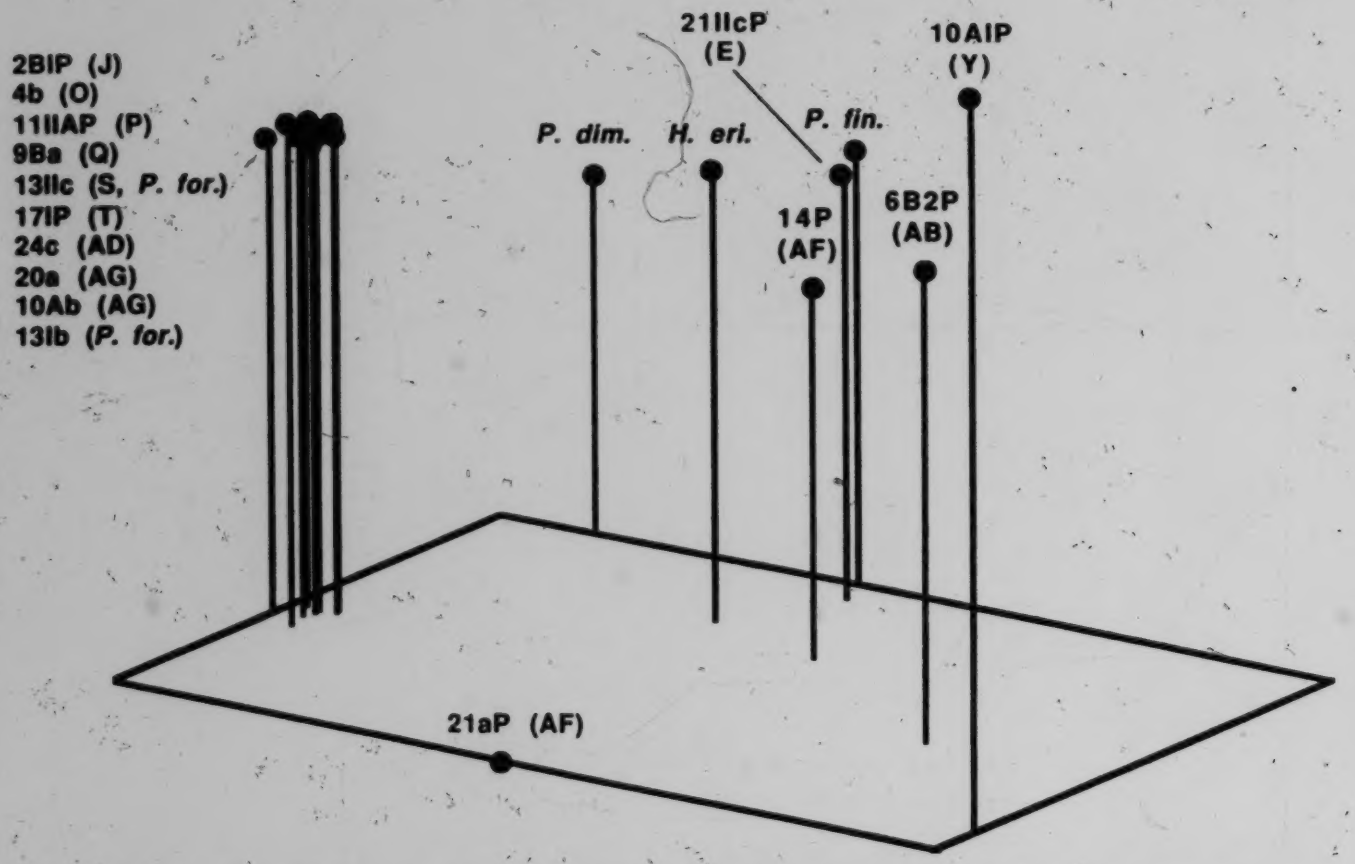

Fig. 3-21. Ordination of correspondence analysis for rDNA restriction bands of 18 isolates restricted with *Alu* 1, *Cfo*, *Msp*, *Rsa* 1, *Hae* III, and *Nde* II (*H. eri.* = *Hymenoscyphus ericae*, *P. dim.* = *Phialocephala dimorphospora*, *P. for.* = *P. fortinii*, *P. fin.* = *Phialophora finlandia*).

conspecific or congeneric with *P. finlandia* since cultural morphologies of the two fungi vary distinctly. A complete data set was not obtained for *Cenococcum geophilum* but available data indicate that the two isolates of *C. geophilum* examined are conspecific and differ greatly from all other isolates tested.

Discussion

Homogeneity of cultural groups

The cultural groups are useful in the delineation of taxa of sterile fungi. For example, all cultural groups displaying the presence of sclerotia were shown to belong to *P. fortinii* by RFLP analysis. However, while the majority of cultural groups belonging to *P. fortinii* have very similar colonial morphology (e.g. cultural groups Q, R, S, T) , there are a few exceptions. For example, 6Ba (X) lacks sclerotia and displays a striking hyphal mat that has feathery, radial branches (Fig. 3-10), yet it is apparently related to *P. fortinii* based on restriction fragment analysis. However, cultures of *P. fortinii* lacking hyphal aggregations will form radial branches similar to 6Ba (X) when grown for 6 months on Oat medium instead of the CMA used as a standard for description of colonial morphology, suggesting that this character is at least partially controlled by nutrient regime. Fine-scale resolution of the *P. fortinii* cluster did not reveal any sub-clustering of isolates based on cultural groups. Differences among the groups belonging to *P. fortinii* therefore do not appear to be reflected in RFLPs.

All the cultural groups that could not be assigned to *P. fortinii*, (i.e. E, Y, AB, AD and AF) are dispersed quite widely in the ordination (Fig. 3-21), suggesting that each of these cultural groups is taxonomically distinct at least at the species level. In general, cultural groups correspond well with grouping based on

molecular characters. Nevertheless, restriction fragment analysis clustered two isolates from the same cultural group far apart (isolates 21aP and 14P of cultural group AF), showing that even the conservative character evaluation used to split isolates into cultural groups was not sufficient to separate all of the taxa.

Restriction analysis indicates that the majority of sterile endophytic fungi isolated from the subalpine site tested are conspecific or closely related to the hyphomycete *Phialocephala fortinii* (eight out of 13 isolates; Fig. 3-21). *Phialocephala fortinii* isolates were obtained from all the dominant dwarf shrubs (*Cassiope mertensiana*, *Phyllodoce empetriformis*, *P. glanduliflora*) and from five of six sub-dominant plant species examined (*Luetkea pectinata*, *Rubus pedatus*, *Vaccinium membranaceum*, *V. scoparium*, and an unidentified graminoid). This study found little evidence for host specificity in *P. fortinii*. Three of the five sterile endophytes not belonging to *P. fortinii* were isolated from sub-dominant hosts (sub-dominant: grass (21IIcP), *L. pectinata* (14P, 21aP), dominant: *P. glanduliflora* (6B2P), *C. mertensiana* (10AIP)).

Restriction fragment analysis of rDNA has been used for distinguishing isolates at the species level. Intraspecific variation in fragment lengths exists, but polymorphisms tend to be considerably more pronounced between species than within species (e.g. Armstrong *et al.* 1989, Kohn *et al.* 1988). Current discussions of the validity of RFLP analysis focus on the numbers of isolates required for reliable analysis (e.g. Hillis and Davis 1988, Williams *et al.* 1988). Ideally, several isolates of each species examined should be used. Also, Rothschild *et al.* (1986) point out that RFLP analysis is complementary to and should always be used in conjunction with morphological data to prevent tautological arguments which define evolutionary distance on the basis of

fragment sequence differences. In our study, *Phialophora finlandia* and isolate 21IIcP (E) appear to represent a confirmation of this observation; while both fungi have similar restriction fragment banding patterns the differences in cultural morphology indicate that the two isolates are not conspecific. The tight clustering of the two *P. fortinii* isolates, and of the other isolates sharing morphological characters with *P. fortinii* observed in our study (Fig. 3-21) reinforces the notion that rDNA variation is diagnostic at the species level.

Definition of cultural groups based on colonial morphology allows splitting of isolates belonging to the same species (here cultural groups P to W, and X) and may occasionally lump isolates apparently belonging to different species (here isolates 14P and 21aP). Yet, it allows grouping of isolates on a preliminary basis and reduces the chaos of numerous isolates to a manageable level. Cultural grouping in combination with restriction fragment analysis can help determine which morphological characters are of taxonomic value, speeding up future work.

In summary, results suggest that taxonomic work, particularly when examining sterile isolates, may benefit greatly both from definition of cultural groups and from restriction fragment analysis.

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4. AXENIC MYCORRHIZAL SYNTHESIS USING *PHIALOCEPHALA FORTINII* AND *MENZIESIA FERRUGINEA* (ERICACEAE)*

Introduction

Dark, grey to black, sterile fungi are common in cold soils and roots and have been designated as *Mycelium radicis atrovirens* Melin (Melin 1922). Some of the dark fungi isolated from the roots of conifers can be induced to form conidia through prolonged incubation at low temperatures. One of these, *Phialocephala fortinii* Wang and Wilcox, is a dark, grey-black hyphomycete which was described originally from the roots of black spruce (*Picea mariana* (Mill.) BSP.) and pine (*Pinus sylvestris* L.) (Richard and Fortin 1973, Wang and Wilcox 1985).

In axenic culture with *Pinus resinosa* Ait. and *Picea rubens*Sarg., *P. fortinii* acts as a pathogen, leading to the formation of stunted and malformed seedlings. The fungus was observed to form densely packed masses in the cortical cells immediately adjacent to the endodermis, as well as in the cells of the endodermis itself. In roots of small diameter, *P. fortinii* invaded the stele and caused extensive damage to stelar tissue (Wilcox and Wang 1987). Recently, *P. fortinii* has been isolated from the roots of a broad taxonomic range of subalpine plants. Eight per cent of isolates obtained from the roots of ericaceous dwarf shrub vegetation were assigned to *P. fortinii* based on the conidial state (Stoyke and Currah 1991). Genetic analysis revealed that two thirds of the sterile isolates obtained belonged to *P. fortinii* (Stoyke et al. in preparation). It appears, therefore, that *P. fortinii* can be a dominant colonizer of roots in subalpine

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* A version of this chapter is being prepared for publication. Stoyke, G. and Currah, R.S. Arctic and Alpine Research: in preparation.

perennials. Yet, the ecological role of the root endophyte in subalpine systems is unknown.

In order to determine the role of *P. fortinii* in subalpine mycorrhizae, we examined the morphology of synthetic associations of *P. fortinii* with *Menziesia ferruginea* J.E. Smith (Ericaceae), a montane shrub, as well as the effects of the fungus on plant growth in axenic cultures. A smaller number of inoculations with a sterile and unidentified, as well as fruiting and identified fungal species, was set up to study the comparative morphology of the root associations.

Materials and Methods

P. fortinii and a sterile white endophyte (isolate G21IIcP) were obtained from a subalpine site in the Canadian Rocky Mountains. The site, in the vicinity of Outpost Lake, Jasper National Park, was sampled on July 26, 1988 and is a dwarf shrub heath dominated by *Cassiope mertensiana* (Bong.) D. Don, *Phyllodoce glanduliflora* (Hook.) Coville, and *P. empetriformis* (Smith) D. Don, and *Abies lasiocarpa* (Hook.) Nutt., at 2010 m a.s.l. Nomenclature of vascular plants follows Packer (1983).

Plants were excavated with their root systems intact and packed in moist paper towels. Within 24 hours all plants were transported to the laboratory where the roots were separated and washed. Root segments five to ten mm in length were submersed in a 20 % solution of household bleach (5.25 % solution of sodium hypochlorite) for one minute, and rinsed twice with sterile, distilled water. These were placed onto modified Melin-Norkrans agar (MMN; Marx 1969). After one week, fungi growing from the root samples were transferred to

corn-meal agar (CMA; Difco, Detroit, USA). After two months all strains were transferred to cereal agar (CER; Padhye *et al.* 1973) and oat-meal agar (OAT; Padhye *et al.* 1973) and incubated in the dark at 4°C. Isolates were examined microscopically and by restriction fragment length polymorphism (RFLP) analysis for identification (Stoyke and Currah 1991, Stoyke *et al.* in preparation). Representative isolates obtained from subalpine habitats were deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH).

Axenic mycorrhizal synthesis was obtained using *Menziesia ferruginea* seeds collected in the vicinity of Tunnel Mountain, Banff, Alberta, *Phialocephala fortinii* Wang and Wilcox isolated from *Luetkea pectinata* (Pursh) Kuntze (Rosaceae) and a variety of other isolates (Table 4-1, Figs. 4-1, 4-4). Seeds of *M. ferruginea* were surface-sterilized using the methods of Smreciu and Currah (1989) and plated on tap-water agar (TWA; 20 g agar, 1 l tap water) until germination. Seedlings at least 4 mm tall were then transferred to CEL (cellulose agar; Warcup 1973) and inoculated with a plug from a fresh culture of fungus immediately after transfer. Three plants were inoculated with *Phialocephala dimorphospora*, *Phialophora finlandia*, *Cenococcum geophilum*, or the sterile, hyaline isolate G21IIcP, respectively (Table 4-1). Fifty plants were inoculated with *P. fortinii,* and 50 plants were grown uninoculated as controls. After three months, plant fresh weights were determined and the whole roots were excised, mounted in glycerin jelly, and examined by bright field and Nomarski microscopy. Differences in seedling mortality were examined by Chi-square analysis and differences in plant weight by t-test using the program StatWorks (Cricket Software, Inc., Philadelphia, Pa., USA).

Table 4-1. Fungal isolates used for mycorrhizal synthesis with *Menziesia ferruginea* (Ericaceae)

Isolate	Host	Location
13IIc(*Phialocephala fortinii* Wang and Wilcox)	*Luetkea pectinata*	Alberta,CDN
21IIcP	Unidentified Gramineae	Alberta,CDN
FAG-15 *(Phialophora finlandia* Wang and Wilcox*)*	*Pinus sylvestris*	Suonenjoki, Finland
DAOM 165556a *(Phialocephala dimorphospora* Kendrick*)*	Decaying wood	Unknown
155, 28 *(Cenococcum geophilum* Fr.*)*	*Pinus virginiana*	Unknown

Results and Discussion

At least 87 % of terrestrial plants form mycorrhizal associations with fungi which enhance plant growth and health. Most of these associations are vesicular-arbuscular mycorrhizae (VAM). VAM are found in a wide variety of plant families and occur in many crop plants. Ectomycorrhizae are typical of temperate forest trees and are formed primarily with basidiomycete fungi. Three % of plant species form this type of association. Also, a number of plants have associations unique to their family, such as the ericoid, orchid and nyctaginaceous mycorrhizae. Fungus-root associations described from alpine sedges may represent yet another mycorrhizal type (Haselwandter and Read 1982, Hudson 1986, Lewis 1987, Lalonde and Piché 1988).

While VA mycorrhizae are ubiquitous in many ecosystems, these associations appear to be less important or lacking in many arctic and alpine or subalpine communities (Bledsoe et al. 1990, Kohn and Stasovski 1990, Haselwandter 1987, Haselwandter and Read 1980, Stoyke and Currah 1991). Bledsoe et al. (1990) examined the roots of 55 species of herbaceous and woody plants in the Canadian high arctic and found no VAM. Kohn and Stasovski (1990) examined 24 species of plants on Ellesmere island (78°53'N, 75°55'W) and found VAM in only one species, *Dryopteris fragrans* (L.) Schott (Polypodiaceae).

Haselwandter and Read (1980) found ericoid mycorrhizae and VAM to be the dominant mycorrhizal types at elevations of up to 2500 m in the European Alps. However, in nival zone vegetation of 3100 to 3200 m elevation, dark-walled septate fungi producing micro-sclerotia and occasional coils in root cortical cells became more dominant than VAM fungi. These fungi could not be identified

since no reproductive structures were formed in culture. Runner hyphae, swollen cells and micro-sclerotia were likened in appearance to *Phialophora* and *Rhizoctonia*, respectively (Read and Haselwandter 1981). Increased dry matter production and increases in shoot phosphorus concentrations of *Carex* inoculated with these fungi were observed (Haselwandter and Read 1982). Shoot phosphorus content increased in both species tested, *C. firma* and *C. sempervirens*, by a factor of approximately six and two, respectively. Significant increases in plant dry weight were observed only in *C. firma*, whose dry weight was five times that of the uninoculated control. The fungi had been isolated from *Carex* spp., and were grown in pots of sterile quartz sand and diluted nutrient solution for three months.

While Kohn and Stasovski (1990) did not observe dark, septate root endophytes, Bledsoe et al. (1990) report dark, septate hyphae on the roots of many plants. Light brown hyphae were found and black hyphae similar in appearance to *Cenococcum geophilum* formed dense mats on some root tips. Bissett and Parkinson (1979a, b, c) examined the fungal flora of soils at three sites on Mt. Allan in Alberta. In contrast to other soil saprophytic fungi, hyaline and dark sterile fungi increased in density at greater sampling depths (15-18 cm vs. 0-2 and 5-8 cm). Dark, sterile fungi were the dominant fungal group at 15-18 cm depth. This distribution pattern could be due to association of the dark soil endophytes with roots. Currah and Van Dyk (1986) examined the roots of 179 vascular plant species of Alberta and found dark, septate fungi on the roots of 6 % of low-altitude and 79 % of alpine, subalpine or montane species.

Dark, septate hyphae were also found on many roots of plants from a subalpine site in Jasper National Park, Alberta (Stoyke and Currah 1991; Figs. 4-

6, 4-8, 4-10 and 4-15). Surface-sterilized roots from 17 plant species yielded several strains of *Phialocephala fortinii*, a dematiaceous fungus previously isolated from roots of *Picea mariana* and *Pinus sylvestris* (Richard and Fortin 1973, Wang and Wilcox 1985). Of 127 isolates obtained, 86 % were dark and 14 % white. All the white endophytes and 90 % of the dark endophytes were sterile. Genetic analysis of representative samples of the sterile isolates indicated that two thirds of these also belonged to *P. fortinii* (Stoyke *et al.* in preparation). *Phialocephala fortinii* therefore appears to represent the major root endophytic fungus that can be isolated on agar medium from the site. Microscopic examination of roots revealed the presence of hyaline hyphae on a number of roots. However, dark hyphae were by far the most common colonizers of root surfaces and root cortical cells (Stoyke and Currah 1991).

In our study, *Phialocephala fortinii* formed distinct associations with *Menziesia ferruginea* in axenic culture. Characteristic configurations of darkly pigmented hyphae were observed on the root surfaces and inside cortical cells. Hyphae formed a loose network on the root surface, typically running parallel to the axis of the root (Fig. 4-5, 4-11). Hyphae were 2-4 µm broad, thick-walled, simple septate, occasionally repeatedly branched and contorted, with cells long and narrow or short, swollen and lobed, and occasionally forming structures resembling sclerotia typically 30 to 50 µm in size. These consisted of irregularly lobed, closely packed hyphae often filling entire cortical cells (Figs. 4-7, 4-9). These structures were similar in morphology to hyphae observed in roots of subalpine dwarf shrub heath (Figs. 4-6, 4-8, 4-10 and 4-15) and remarkably similar in appearance to associations observed in the Austrian Alps (Read and Haselwandter 1981).

Figs. 4-1to 4-4. Mycorrhizal synthesis of *Menziesia ferruginea* (Ericaceae) with *Phialocephala fortinii* on sterile cellulose agar dishes. X 0.95.

Figs. 4-1. Inoculated seedling after three months.

Figs. 4-2. Three month old seedling contaminated with *Penicillium* sp.

Figs. 4-3. Sclerotia formed by *Phialocephala fortinii* after three months of growth in cellulose agar.

Figs. 4-4.Uninoculated seedling after three months.

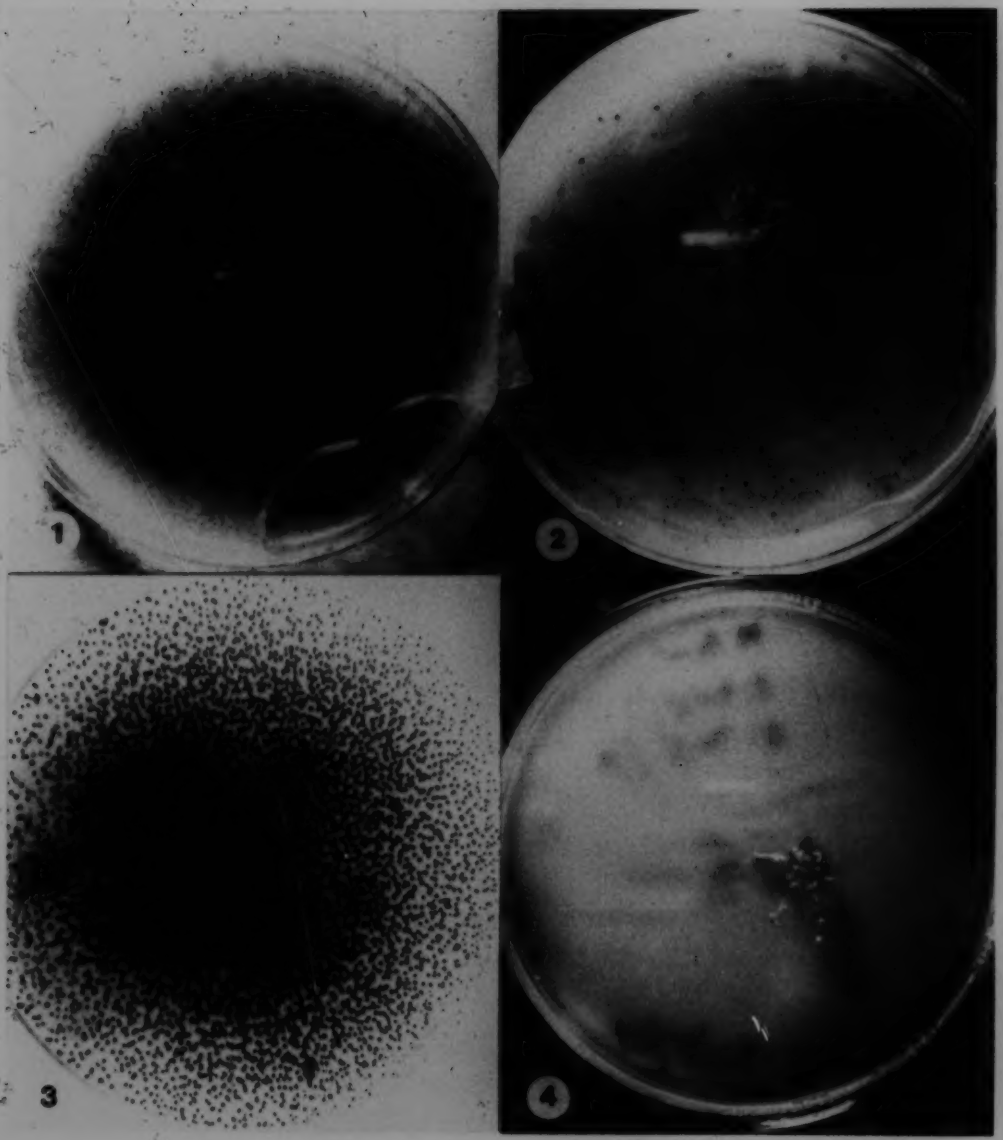
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Fig. 4-5. Network of swollen and repeatedly branched hyphae (arrow) of *Phialocephala fortinii* on roots of *Menziesia ferruginea* after three months of growth on cellulose agar. X 500.

Fig. 4-6. Swollen hyphae (arrow) on root cortical cells of subalpine *Cassiope mertensiana* from Jasper National Park, Alberta. X 500.

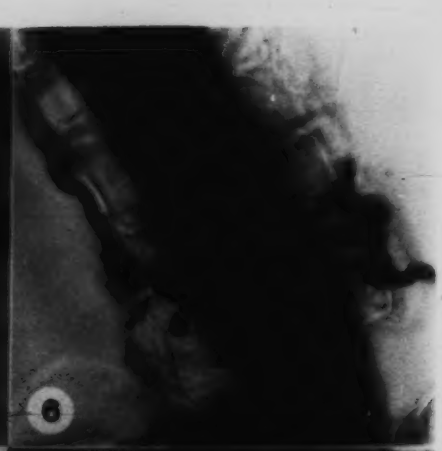
Fig. 4-7. Cortical sclerotium (arrow) of *Phialocephala fortinii* on roots of *Menziesia ferruginea*. X 500.

Fig. 4-8. Cortical sclerotia (arrows) of a dematiaceous, septate fungus on roots of *C. mertensiana* from Jasper National Park, Alberta. X 500.

Fig. 4-9. Two intracellular sclerotia (arrow) of *Phialocephala fortinii* filling cortical root cells of *Menziesia ferruginea*. Size of each cell/sclerotium approximately 20 by 50 µm. X 500.

Fig. 4-10. Intracellular hyphae of a dematiaceous, septate fungus on roots of *Vaccinium membranaceum* from Jasper National Park, Alberta. Note compression of hyphae passing through anticlinal cortical cell wall (arrow). X 500.

5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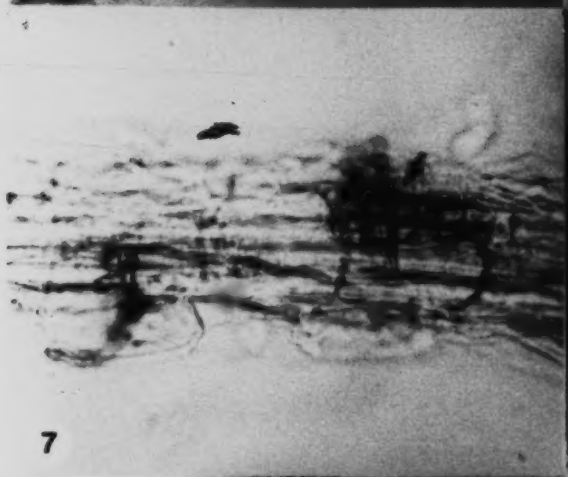
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Fig. 4-11. Swollen and branched hyphae of *Phialocephala fortinii* representing early stages of sclerotia development (arrow) on roots of *Menziesia ferruginea* after three months of growth on cellulose agar. X 480.

Fig. 4-12. Sclerotium-like conglomerations of hyphae (arrow) of *Phialocephala dimorphospora* on roots of *M. ferruginea*. X 480.

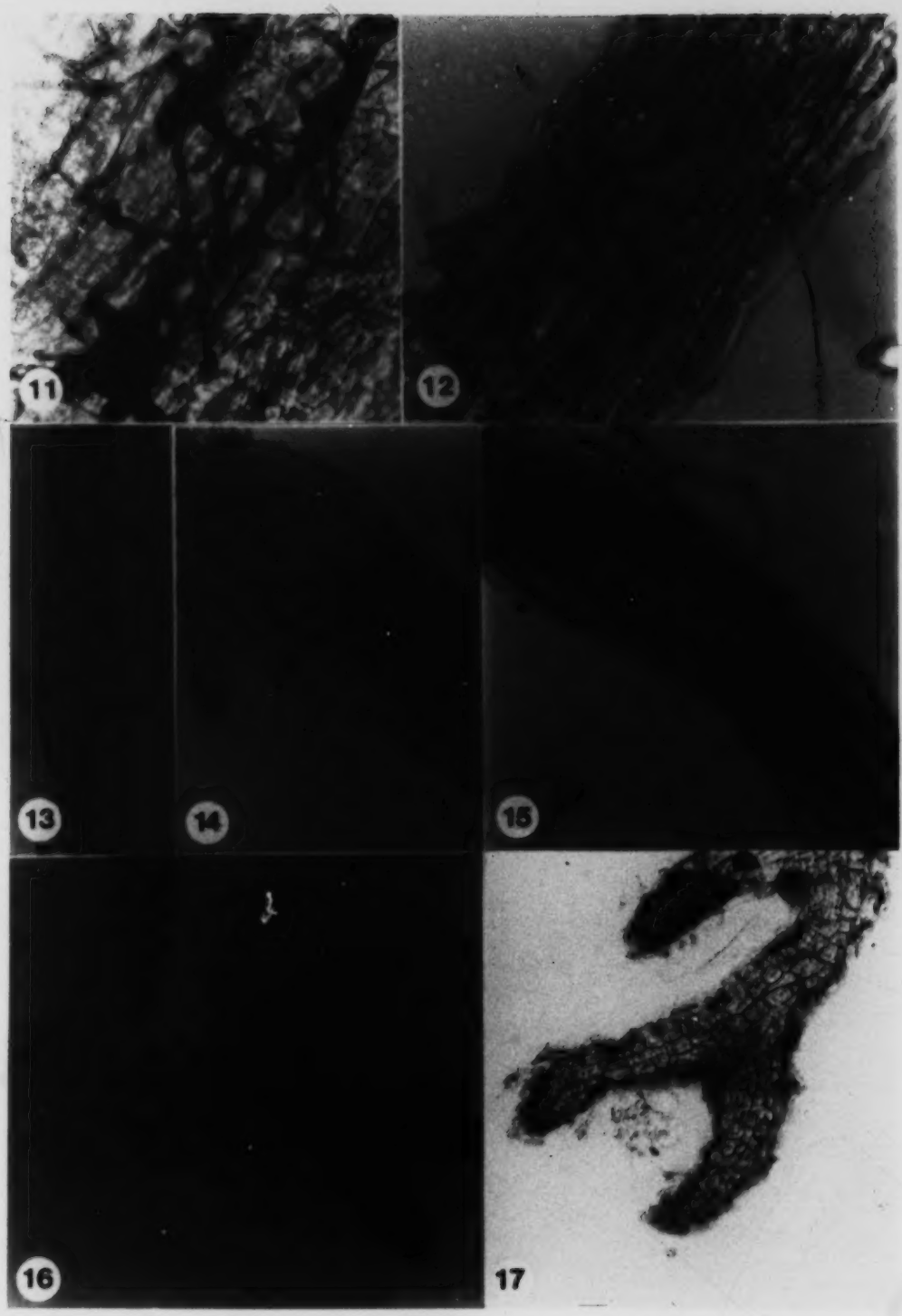
Fig. 4-13. Extracortical hyphae (arrow) of *Cenococcum geophilum* on roots of *M. ferruginea*. X 480.

Fig. 4-14. Hyphal network (arrow) of *Cenococcum geophilum* on roots of *M. ferruginea*. X 120.

Fig. 4-15. Extracortical network (arrow) of dematiaceous, septate hyphae on roots of *Vaccinium membranaceum* from Jasper National Park, Alberta. X 250.

Fig. 4-16. Root of *M. ferruginea* grown in the presence of sterile white endophyte G21IIcP isolated from Jasper National Park, Alberta, displaying stunted root growth (arrow) . X 120.

Fig. 4-17. Uninoculated root of *M. ferruginea*. X 120.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A number of known fungi were grown with *M. ferruginea* for comparison with *P. fortinii* associations. *Cenococcum geophilum* is a dark, septate fungus forming ectomycorrhizae with subalpine trees (Trappe 1988). In our study, *C. geophilum* formed loose wefts of hyphae on root surfaces (Fig. 4-14) but rarely penetrated the root cortex (Fig. 4-13). Hyphae of *C. geophilum* are similar to those of *P. fortinii*, however, *C. geophilum* did not form any type of sclerotium. Growth habit of the hyphae on the root surface resembled associations in field samples (Fig. 4-15), but *C. geophilum* had not been isolated from the subalpine site. *Phialocephala dimorphospora* is congeneric with *P. fortinii* and has also been isolated from conifer roots. *P. dimorphospora* showed little hyphal development on roots of *M. ferruginea*. Formation of a sclerotium-like structure was observed in one case (4-12), but no intracortical sclerotia were formed. *Phialophora* has been likened in appearance to hyphal structures on the roots of alpine plants described by Read and Haselwandter (1981). In our study, *Phialophora finlandia* did not form any association with *M. ferruginea*. Roots of plants inoculated with *P. finlandia* were similar in appearance to uninoculated plants. The sterile, white endophyte G21IIcP was isolated from a graminoid at the same subalpine site as *P. fortinii*. Very few hyphae were observed on the roots of *M. ferruginea* seedlings grown with this isolate. Instead, the seedlings showed increased deposition of phenolics in cortical cells and displayed stunted root growth typical of pathogenic relationships (Fig. 4-16). Based on these preliminary tests of fungi not belonging to *P. fortinii*, only *C. geophilum* displayed associations which appear potentially mycorrhizal. Associations of *C. geophilum* and *P. fortinii* could be distinguished easily in culture by the lack of sclerotial development in *C. geophilum*.

Wilcox and Wang (1987) found *P. fortinii* to be pathogenic in *Pinus resinosa*

and *Picea rubens*. Root cross sections revealed heavy colonization of the cortex and endodermis and extensive damage to stelar tissue, particularly in smaller roots. O'Dell et al. (1990) report no degradation of host tissue by *P. fortinii* growing with *Pinus contorta*, a montane species of pine, or *Lupinus latifolius*. Colonization of cortical cells occurred, but stellar tissues were not invaded.

P. fortinii increased seedling mortality by a magnitude of 10 in our study (Table 4-2). Dead seedlings were of approximately the same size as the seedlings before inoculation, indicating that seedling death due to the presence of the fungus occurred only in very young plants. However, growth of established plants was not significantly affected by the presence of *P. fortinii*(Table 4-3). *P. fortinii* did not appear to invade stellar tissue in *M. ferruginea* and no pathogenic effects were observed in established plants. Pure cultures of *M. ferruginea* with *P. fortinii* on an agar medium grown at room temperature may not be ideal for testing plant growth response. Cellulose agar provides a relatively ready source of carbohydrates for *P. fortinii* while offering few nutrients for *M. ferruginea*. Lack of competition from other soil fungi enables *P. fortinii* to develop rapidly in the dish and to physically overgrow *M. ferruginea*. Since uninoculated plants showed no adverse health effects, nutrient supply may not yet have been severely limiting and the addition of the fungus did not represent an advantage to the plant at this stage. While petri dish culture is well suited to microscopic observation of the morphology of the fungus-plant association, future studies of this relationship may benefit from imitating natural conditions as closely as possible. Fungi and plants on (possibly non-sterile) soils containing coarse organic materials and grown at low temperatures may represent a more suitable environment to examine plant growth response under laboratory conditions. Plants infected accidentally with *Penicillium* sp. through aerial contamination

Table 4-2. Mortality of seedlings of *Menziesia ferruginea* grown in the presence and absence of *Phialocephala fortinii* after three months of growth.

	Uninoculated	Inoculated	Probability level*
Total no. plants	27	38	
Dead	1	15	$P < 0.01$

* Significance level based on Chi-square analysis.

Table 4-3. Shoot fresh weights of three month old seedlings of *Menziesia ferruginea* grown in the presence and absence of *Phialocephala fortinii*.

	Uninoculated	Inoculated	Probability level*
n	26	23	
Mean weight (mg)	24.0	21.7	N.S.

* Significance level based on t-test.

Table 4-4. Shoot fresh weights of three month old seedlings of *Menziesia ferruginea* grown in the presence and absence of *Penicillium* sp.

	Uninfected	Infected	Probability level*
n	26	3	
Mean weight (mg)	24.0	78.0	$P < 0.001$

* Significance level based on t-test.

showed significantly increased growth (Table 4-4) The lack of root association of *Penicillium* with *M. ferruginea* (Fig. 4-2) indicates that this is not a mycorrhizal association. Solubilization of nutrients due to breakdown of nutrient agar by *Penicillium* might explain the increased growth response of the seedlings.

P. fortinii appears to form unique associations with perennial herbs and shrubs in subalpine communities. These are characterized by loose hyphal wefts on root surfaces, cortical penetration, and the formation of intracortical sclerotia, densely packed masses of hyphae filling entire cortical cells. These associations resemble those reported from alpine plants of the European alps and ascribed to fungi resembling *Phialophora* (Read and Haselwandter 1981). Since *Phialocephala fortinii* is the only identified fungal component of this association, I propose the term "P-type" infection for the distinct type of fungus-root association described above. While a mycorrhizal nature of the relationship could not be demonstrated in this study, the apparently widespread occurrence of *P. fortinii* in the roots of subalpine plants at least in some sites indicates that this association may play an important ecological role in subalpine ecosystems.

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5. GENERAL DISCUSSION AND CONCLUSIONS

Summary

1.) One-hundred-eighty-one dark and 13 white endophytes were isolated from the roots of 64 alpine and subalpine herbs and shrubs belonging to 17 species.

2.) . Of the 194 isolates, 181 were sterile. The 13 fruiting isolates belonged to two fungal species, *Oidiodendron griseum* (2 isolates) and *Phialocephala fortinii* (11 isolates). Fruiting could only be induced in *P. fortinii* through incubation on oat or cereal medium for 3 to 6 months at 4°C.

3.) With the exception of the two specimens of *Loiseleuria procumbens* from which *Oidiodendron griseum* was isolated (Site 2), ericoid mycorrhizae were not observed in the roots of ericaceous plants, despite the dominance of ericaceous dwarf shrubs at the sites studied.

4.) Strains of *P. fortinii* producing conidia in culture were isolated from roots of *Cassiope mertensiana, Vaccinium scoparium, Arctostaphylos uva-ursi* (Ericaceae) and *Luetkea pectinata* (Rosaceae).

5.) Hyphae consistent in morphology with *P. fortinii* formed loose hyphal wefts on root surfaces and characteristic sclerotia in cortical cells of many roots collected in the field.

6.) Sterile fungi were placed in 33 "cultural groups" based on morphologies of cultures grown on corn meal agar (CMA) for 3 months.

7.) Ribosomal DNA of 13 sterile fungi from representative cultural groups was extracted and amplified using the polymerase chain reaction (PCR).

Amplified DNA was restricted using six endonucleases. After electrophoretic separation, restriction fragments were scored for presence/absence. Based on correspondence analysis, eight of the 13 sterile isolates were found to belong to *P. fortinii.* The remaining five isolates clustered independently and presumably represent distinct species.

8.) Sterile isolates of *P. fortinii* were obtained from *Phyllodoce empetriformis* and *P. glanduliflora* (Ericaceae) as well as from the species listed in 4.)

9.) *P. fortinii* displays a range of culture morphologies. Not all isolates belonging to *P. fortinii* displayed the presence of sclerotia, but all cultures displaying formation of sclerotia belonged to *P. fortinii.* Submerged hyphal mats were pale to dark brown and distributed evenly, radially or concentrically zonate, or grouped in radial branches. However, all cultures were characterized by the formation of mouse-grey (grey to brown) aerial hyphae.

10.) Axenic inoculation of *Menziesia ferruginea* with *P. fortinii* on cellulose agar plates resulted in the formation of loose hyphal wefts on the roots and characteristic intracellular cortical sclerotia similar to those observed in field material.

11.) Inoculated plants displayed a significant, ten-fold increase in seedling mortality.

12.) Plant growth rates after 3 months were not significantly affected by inoculation.

13.) *P. fortinii* did not invade the stele nor were any other signs of pathogenicity observed in roots.

General Discussion and Conclusions

Vesicular-arbuscular mycorrhizae (VAM) are the dominant form of mycorrhiza on earth. VAM are found in most mycorrhizal herbs, shrubs, and some trees. However, it appears that VAM are less important in arctic and alpine tundra and subalpine communities. For example, Read and Haselwandter (1981) found that VA fungi colonized an average of 20 % of roots of European alpine plants at 1600 m elevation and only 7 % at 3200 m. Kohn and Stasovski (1990) studied 24 species of plants on Ellesmere Island (arctic Canada). Only a single species was reported to have VA colonization. In a survey of 55 plant species on Devon Island (NWT, Canada), Bledsoe et al. (1990) found no evidence of VAM. In our study of the nine dominant herbs and ericaceous dwarf shrub species of a subalpine meadow at 2010 m elevation in Jasper National Park, Canada, no VAM were observed in root preparations (Stoyke and Currah 1991/Chapter 2).

Read and Haselwandter (1981) occasionally found ectomycorrhizal associations along the 1600 - 3200 m altitudinal range which they studied. Also, in a number of sites the "fine endophyte" (*Glomus tenuis*) was observed. However, in a number of plant communities at elevations above 2000 m, dark septate hyphae represented the dominant form of root infection. Stoyke et al. (Chapter 3) found that *Phialocephala fortinii* represented 13 out of 18 representative isolates, based on genetic analysis. This fungus forms dark, septate hyphae on plant roots. *P. fortinii* as well as the dark, septate fungi observed by Haselwandter and Read (1981) form characteristic sclerotia (conglomerations of irregular lobed hyphae) in root cortical cells. On the basis of cultural morphology and the formation of these characteristic sclerotia we believe that the dark isolates from the European alps may be congeneric or conspecific

with *P. fortinii*. Since *P. fortinii* is the only identified isolate of this form of root colonization we propose the term "P-type" infection for this predominantly alpine and subalpine association.

The ecological role of P-type fungi in subalpine ecosystems is unclear. Haselwandter and Read (1982) found increases in shoot phosphorus concentrations and enhanced growth in mycorrhizal syntheses with *Carex* species. *P. fortinii* grown axenically with *Menziesia ferruginea* caused a ten-fold increase in seedling mortality but did not affect plant growth rates. Root preparations did not reveal any pathogenic effects of *P. fortinii* on *Menziesia ferruginea*. More detailed studies of the eco-physiological role of P-type fungi are now required to assess their role in alpine and subalpine communities. Laboratory experiments should strive to simulate subalpine weather and soil conditions closely. Subalpine soils, rather than artificial media, may more closely reflect nutrient levels typical of subalpine environments. Finally, non-sterile soils may yield results which cannot be observed under sterile conditions. For example, competition with edaphic fungi may prevent overgrowth of *Menziesia ferruginea* seedlings by *P.* fortinii, or *P. fortinii* may benefit plant growth through synergistic effects with other components of the soil microflora.

References

Bledsoe, C., Klein, P., and Bliss, L.C. 1990. A survey of mycorrhizal plants on Truelove Lowland, Devon Island, N.W.T., Canada. Can. J. Bot. 68: 1848-1856.

Haselwandter, K. and Read, D.J. 1982. The significance of a root-fungus association in two *Carex* species of high-alpine plant communities. Oecologia 53: 352-354.

Kohn, L.M., and Stasovski, E. 1990. The mycorrhizal status of plants at Alexandra Fiord, Ellesmere Island, Canada, a high arctic site. Mycologia 82: 23-35.

Read, D.J. and Haselwandter, K. 1981. Observations on the mycorrhizal status of some alpine plant communities. New Phytol. 88: 341-352.

APPENDIX 1: COMPOSITION OF CULTURE MEDIA

Warcup's Cellulose Medium (CEL)

Sodium Nitrate	0.3 g
Potassium Dihydrogen Phosphate	0.2 g
Magnesium Sulphate	0.1 g
Potassium Chloride	0.1 g
Yeast Extract	0.1 g
Distilled Water	1000.0 ml
Cellulose (Type 20, Sigmacell)	10.0 g
Agar (Bacto-, Difco)	12.0 g

Reference: Warcup, J.H. 1973. Symbiotic germination of some Australian Orchids. New Phytol. 72: 387-392.

Oatmeal Agar

Rolled Oats	7.0 g
Magnesium Sulphate	0.7 g
Potassium Phosphate	1.05 g
Sodium Nitrate	0.7 g
Agar	12.6 g
Water	700.0 ml

Reference: Weitzman, I. and Sliva-Hunte, M.. 1967. Sab. 5:335-340

Modified Melin-Norkrans Medium (MMN)

Glucose	5.0 g
Yeast Extract	1.0 g
Malt Extract	2.0 g
Potassium Phosphate, mono- or dibasic, 10 % solution	5.0 ml
Calcium Chloride, 1 % solution	2.5 ml
Ammonium Phosphate, monobasic, 10 % solution	2.5 ml
Magnesium Sulphate, 10 % solution	1.5 ml
Ferric Chloride, 1 % solution	1.2 ml
Bacterial Agar (Difco)	15.0 g
Distilled Water	1000.0 ml
Antibiotics: Streptomycin Sulphate, 100 ml solution contains 0.5 g	6.0 ml
Tetracycline (Nova), 0.25 g/100 ml	0.8 ml

Reference: Marx, D.H. 1969. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopath. 59: 153-163. With some modifications.

Pablum Cereal Agar

Mead Johnson Mixed Cereal Pablum	50 g
Bacto-Agar (Difco)	9 g
Water	500 ml

Reference: Padhye, A.A., Sekhon, A.S., and Carmichael, J.W. 1973. Ascocarp production by *Nannizzia* and *Arthroderma* on keratinous and nonkeratinous media. Sabouraudia 11: 109-114.

Cornmeal Agar

Cornmeal Agar Mix (Difco)	17.0 g
Water	1000.0 ml

Autoclave at 121°C for 20 min. Pour into petri dishes (makes ~ 37 plates). Let cool for ~ 1hour (until jello-like) and leave on counter for 24 hours for some of the condensate to disappear. Label: type of medium and date.

Reference: Cornmeal agar, Difco, Detroit, USA.

Cereal Agar (CER)

Pablum	50 g
Agar	10 g
Water	500 ml

Reference: Padhye, A.A., Sekhon, A.S., and Carmichael, J.W. 1973. Ascocarp production by *Nannizzia* and *Arthroderma* on keratinous and nonkeratinous media. Sabouraudia 11: 109-114.

Tap Water Agar

Agar	20.0 g
Water	1000.0 ml

Reference: Currah, Smreciu, Hambleton, Sigler: AERT T01014

APPENDIX 2: MOLECULAR PROTOCOLS (rDNA ISOLATION, AMPLIFICATION AND RESTRICTION)

DNA Extraction:

Total DNA -CTAB m/p

1. Grind lyophilized tissue in mortar with a small quantity of liquid nitrogen. Clean mortar with ethanol.
2. Add approx 100 mg dry powder to 1.5 ml microcentrifuge tube (represents 2/3 of conical portion of tube). Add 700 µl of CTAB Extraction buffer (700 mM NaCl, 50 mM Tris, pH 8, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% CTAB, 0.2 % mercaptoethanol) to tube and mix briefly to produce a homogeneous solution. (1% PVPP may be added if pigments present).
3. Incubate mixture at 60 C for 45 min.
4. Emulsify mixture by adding 700 µl of Chloroform:Octanol (C:O) then "finger vortex".
5. Centrifuge (13000 rpm, 15 min, 25°C). Transfer upper aqueous phase to new 1.5 mL microcentrifuge tube, being careful not to transfer any of the cell debris phase!
6. Precipitate the nucleic acid by adding an equal or greater volume of isopropanol and inverting to mix. Usually a large nucleic acid "rope" will come out of solution immediately; if not let solution stand at room temperature for 10 min.
7. Centrifuge (6500 rpm, 3 min, 25°C) and pour off supernatant, being careful not to loose pellet! If pellet is tight, invert tubes on lab wipes and let them drain (if pellet is not tight, then pipette off supernatent with micropipette and skip draining step).
8. Resuspend pellet in 300 µl of TE-8 (10mM Tric-Cl pH 8.0, 1 mM EDTA). To aid

resuspension you can heat to 60°C for 10 min followed by gentle finger vortexing.

9. Digest RNA with 1 µl RNase A (10 mg/mL) at room temp for 1 hr.
10. Emulsify by adding an equal volume of C:O, finger vortex, centrifuge (13000 rpm, 5 min, 25°C) and transfer the aqueous phase to fresh tubes.
11. Precipitate DNA by adding 50 µl of 7.5 M ammonium acetate (the final concentration is 1.5 M) to the aqueous phase followed by 1 ml of cold ethanol. Mix each tube immediately after addition of the ethanol. Usually a "rope" of DNA forms immediately, if not, let the mixture sit at -20°C for 20 min.
12. Centrifuge (6500 rpm, 3 min, 25°C), discard the supernatant, and invert the tubes for a few minutes to drain the pellet.
13. Resuspend the pellet in 300 µl of 200 mM Ammonium Acetate. To aid resuspension you can heat to 60°C for 10 min followed by gentle finger vortexing.
14. Precipitate DNA by adding 1 mL of cold ethanol. Mix each tube immediately after addition of ethanol. Usually a "rope" of DNA forms immediately, if not, let the mixture sit at -20°C for 20 min.
15. Centrifuge (6500 rpm, 3 min. 25°C), discard supernatant, and invert tubes for a few minutes to drain the pellet. Wash pellet with 1 mL of 70 % EtOH (centrifuge). Dry pellet for 10 min in vacuum oven. Add 50 µL of TE-8 and resuspend by incubating overnight at 4°C or, if time is short, incubating at 60°C for 10 min.

Storage: It is best to finish the whole isolation in one day, and use the DNA the next. However, it may be stored at -20°C for short periods (but should not be removed often because freezing-thawing shears the DNA). It may be stored at -80°C indefinitely.

Reference: Zolan, M.E., and Pukkila, P.J. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. 6: 195-200. Modifications by Keith Egger, pers. comm.

PCR Protocol

1. PCR reaction mixture:

10 µl amplification buffer
16 µl tNTP (nucleotides)
62 µl PCR water (twice glass-distilled, deionized, autoclaved)
5 µl primer "A"
5 µl primer "D"
0.5 µl Taq polymerase

2. Add two drops of sterile mineral oil to seal mixture.

3. Add 1 to 5 µl of DNA extract (sample).

4. Add one drop of oil to outside of tube for better seal.

5. Insert into PCR machine (e.g. "Programmable Thermal Controller, MJ Research Inc").

6. Program cycle as follows:

A.

Temperature (°C)	Time (min)
94	3
52	1
72	4
94	1
52	1
72	4

Run cycle three times.

B.

Temperature (°C)	Time (min)
94	1
50	1

72	4

Run cycle 23 times.

C. Temperature (°C)	Time (min)
94	1
50	1
72	10

(Total of 30 cycles.)

D. Cool to 4°C.

Amplifies DNA approx. 10^6 times. Yields 6-8 µg DNA.

Test DNA Run

1. Cut 4" strip of wide parafilm.
2. Place 1 µl of dye on parafilm for each sample.
3. Add 5 µl of sample to each dye spot, mix carefully, and leave tip near sample.
4. Add 2 µl of lambda marker to 6th dye spot (e.g. if running 11 samples).
5. Pour agarose gel (this could be done before step 1. to save time):
   - put agarose into microwave oven to melt (1 min, another 30 sec if not melted).
   - use stopper to seal gel frame with agarose.
   - cool agarose to hand-warm to prevent warping of frame.
   - pour gel.
6. When gel is solidified, remove well plastic carefully.
7. Load wells.
8. Put lid onto gel frame.
9. Run at 70 V, > 200 mA for 1 to 2 h.

DNA Restriction

1. DNA restriction mixture:

   2 µl buffer
   2 µl spermidine IBSA
   15 µl DNA
   1 µl restriction endonuclease

2. Incubate at 37°C for 3-5 h.

3. Add marker.

4. Load gel.